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Estrogen and the Aging Brain of Male Rats

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Estrogen and the Aging Brain of Male Rats

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Dedication

My parents and my family, who have always supported me and believed I could do this.

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Estrogen and the Aging Brain of Male Rats

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Gonadal steroid hormones exert an influence on many aspects of neurobiology in men, including memory, learning and sexual dysfunction. Though testosterone is the main circulating gonadal steroid hormone in males, estradiol is also important, and together these hormones play complementary roles. While the specific roles of estrogen have been studied to some extent in young adults, little is known during aging, when sexual behavior can become impaired. I used a rodent model to examine estradiol's role in sexual behavior and gene expression in 3 regions, selected for their importance in behavioral neuroendocrine functions and high concentrations of estrogen receptors: the medial preoptic area (mPOA), medial amygdala (MeA), and bed nucleus of the stria terminalis (BnST). My studies focused first on how age and sexual experience affects expression and activation of estrogen receptor α (ERα) and androgen receptor (AR) after sexual behavior in aging intact males. Quantification of neurons expressing hormone receptors in the mPOA revealed that neither ERα nor androgen receptor (AR) showed an age-related change in expression in the mPOA. While both ERα and AR were activated after copulation, the age-related changes were specific to ERα in the central mPOA. There were only mild deficits in sexual behavior. Serum estradiol was also elevated in

both aged and copulating animals, but estradiol concentrations only correlated with sexual behavior in aged animals. In a second study, I determined how hormone deprivation (castration) and replacement with estradiol caused changes to gene expression in the mPOA, BnST and MeA. Each region had unique patterns of gene expression in response to aging and estradiol treatment. The mPOA only had changes in expression as a result of hormone administration, while the BnST had primarily age-related changes. The MeA had the greatest number of affected genes, mainly interactions between estradiol treatment and aging. These studies emphasize the importance of estradiol in aging males, and the need for continued study on its role in neuroendocrine and sexual function.

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Chapter 1: General introduction

1.1 Aging in Men

With aging, males experience decreased levels of circulating sex steroid hormones and experience less sensitivity to these hormones. Age-related changes in hormones have been linked to a variety of symptoms, such as changes in mood, memory, stress response, and sexual function (Bimonte-Nelson et al. 2010, Conrad et al. 2010, Bergendahl et al. 1998, Walf et al. 2009). Most research in aging men has focused on testosterone, the main circulating hormone in males. However, there is evidence that 17β-estradiol, a metabolite of testosterone, also plays a substantial role in these deficits. Here, I review studies examining the production of estradiol during aging in males, and evidence for age-related changes in the function of 17β-estradiol in the brain.

1.2 Aging Impacts Steroid Hormone Production

Serum testosterone, produced by Leydig cells in the testes, is the main source of estradiol in males, as it provides the substrate for conversion to estradiol. Estradiol levels change in response to peripheral manipulation of testosterone (Veldhuis et al. 1994, Lakshman et al. 2010). High levels of testosterone in humans suppress the activity of GnRH neurons in the hypothalamus, leading to a reduction in serum LH, and decreased testosterone production. Conversely, low testosterone stimulates GnRH release in the pituitary, causing LH to be released into the blood stream and stimulate testosterone production by Leydig cells. This is markedly different from the female reproductive system, where estradiol feedback changes from positive to negative during the menstrual

cycle. In humans, both GnRH and LH have pulsatile release patterns in males, with approximately 6-10 pulses occurring over the course of 24 hours (Veldhuis et al. 1994). In aged men, usually defined as men over the age of 60, these rhythms are disrupted, although reports on the exact nature of this disruption vary. Some observations of blunted LH rhythmicity as a function of aging are noted in both men and rats, with pulses becoming less frequent and more irregular (Bergendahl et al. 1998, Veldhuis et al. 1994, Gruenewald et al. 2000). On the other hand, some studies report increased pulse frequency in aged males (Veldhuis et al. 2001, Bonavera et al. 1997). In addition to changes in LH burst frequency, there are also age-related changes in pulse duration and amplitude. Veldhuise (2001) reports that while pulse frequency decreases, pulse duration increases in humans. However, even this increased duration is not sufficient to increase the total amount of LH released, as burst mass decreases with age, and so does amplitude (Hayes et al. 2000, Veldhuis et al. 2005).

Like testosterone, estradiol also exerts effects on the HPG-axis. In addition the gonads, serum estradiol can also come from aromatization in the adrenal glands and adipose tissue (Gautier et al. 2013, Vermeulen et al. 2002). Conversion in adipose tissue increases in older men, possibly accounting for the observation that though serum testosterone declines during aging in men, estradiol often does not (Gates et al. 2013f). Human studies indicate that estradiol influences LH release both at the level of the hypothalamus (pulse frequency) and pituitary gland (amplitude and duration) (Hayes et al. 2000, Mulligan et al. 1999). Exogenous estradiol inhibits LH release, affecting both basal LH, and LH pulse frequency and amplitude (Veldhuis et al 2001, Gooren et al.

1989). Many of these effects of estradiol administration mirror those of aging; namely, estradiol decreases basal LH, pulse frequency (Rochira et al. 2006), and pulse amplitude (Hayes et al. 2000). This effect may be mediated via ERalpha, since an ERalpha antagonist increased pulse frequency in men (Liu et al. 2009). There is also evidence that estradiol reduces sensitivity to GnRH (Hayes et al. 2000). This reduction in sensitivity to GnRH has been seen in some studies of older men (Keenan et al. 2011), other studies did not find this reduction (Iranmanesh et al. 2010). While it is unclear whether estradiol is directly regulating GnRH neurons (which express ERbeta in rats and humans) or whether it is influencing cells that project to GnRH neurons, it is clear that negative feedback changes in older males (Hrabovszky et al. 2001, Hrabovszky et al. 2007). This change, specifically a blunted and attenuated LH surge, may be due to changes in the cells regulating GnRH/LH release, or an increased influence of estradiol due to changes in the testosterone/estradiol ratio in circulation.

In addition to conversion from testosterone to estradiol in the periphery, testosterone may also be converted in the brain by aromatase, creating higher local levels of estradiol that do not enter the blood stream (Vermeulen et al. 2002). For example, in the hippocampus of male mice, estradiol was measured at 8nM/mL, approximately six-fold higher than serum levels (Ooishi et al. 2012). For this reason, serum estradiol levels do not provide a complete picture of changes in the availability of estradiol, and rapid activation/inactivation of aromatase allows for a significant and transient increase in estradiol (Raven et al. 2006, Janowsky et al. 2006, Charlier et al. 2010). There is also evidence that higher levels of estradiol are needed in specific brain regions for

stimulating behaviors, such as sexual behavior and some spatial memory tasks (Cornil 2010, Foster 2012)

Aromatase is widely distributed in the brain. In male rats it is highly expressed in the hypothalamus, hippocampus, cortex and spinal cord (Pietranera et al. 2011). Aromatase production in multiple regions of the CNS, including the preoptic area (POA), hypothalamus (HYP), and amygdala (AMY) is upregulated by testosterone, dihydrotestosterone (DHT), or estradiol (Roselli et al. 1993, Chambers et al. 1991, Zhao et al. 2008). In addition, there are age-related changes in aromatase expression and activity in the POA and HYP (Vermeulen et al. 2002, Roselli et al. 1993, Dellovade et al. 1995). Aged rats showed decreased aromatase in the hypothalamus, but when exogenous testosterone was administered to 4 month old and 12 month old castrates aromatase increased in both groups (Roselli et al. 1993). However, although aromatase expression in both older rats and humans decreases, at least in humans the enzymatic activity of aromatase increases, possibly to compensate for the lowered expression (Vermeulen et al. 2002). While these changes in expression and activity have been noted in both the hippocampus and hypothalamus caution should be exercised in generalizing across nuclei, as changes may be region specific.

While ERalpha or ERbeta may have a great influene in controlling the expression of certain behaviors, it is likely that they interact in subtle ways depending on receptor expression levels and estradiol availability. ERalpha has a higher binding affinity than ER beta, and ERalpha and aromatase knockout mice have more significant impairments in sexual behavior and cognition than ERbeta knockouts (Simpson 1998, Morissette

2008). ERalpha stimulation results in the activation and transcription of different proteins compared to ERbeta, though both alpha and beta knockouts have impairments in estradiol mediated synaptic activity (Fugger et al. 2001)

The distribution patterns of ERalpha and ERbeta are heavily overlapping. However, the ratio of their expression differs by region, indicating that receptor type, not only estradiol sensitivity, is important for behavior. ER alpha is the predominantly expressed receptor in the preoptic area, the bed nucleus of the stria terminalis (BnST), and the ventromedial hypothalamus (VMH). While in other areas, such as the paraventricular nucleus (PVN), ERbeta outnumbers ERalpha, or the two receptors are expressed in approximately equal numbers, as is the case in the anterior hypothalamus and supraoptic nuclei (Scott et al. 2000, Perez et al. 2003, Orikasa et al. 2002, Kudwa et al 2004). There is limited data describing how aging changes receptor expression in males. However, we know that during aging ERalpha either increases, or remains statistically the same but has a trend toward increasing, in the POA, while ERbeta mRNA decreases in the cerebral cortex, hippocampus and amygdala (Wu and Gore 2009, Yamaguchi and Yuri, 2012).

It is important to consider the ratio of ERalpha to ERbeta, not merely their absolute expression (Matthews and Gustafsson, 2003). ERbeta appears to mediate estradiol anxiolytic properties, while ERalpha facilitates sexual behavior in the preoptic area and spinogenesis in the hippocampus (Walf et al. 2008, Russell et al. 2012, Mukai at al. 2010). In other areas, ERbeta activation opposes ERalpha, either by interfering with ERalpha's regulation of certain promoters and response elements, or forming heterodimers with ERalpha to change the magnitude of transcription levels (Matthews

and Gustafsson, 2003, Nilsson and Gustafsson 2000, Peterson 2000, Zhao 2007). ERbeta activation may also down regulate ERalpha expression in specific brain areas, such as the preoptic area, amygdala, and cortex, as well as decreasing spine density in the hippocampus while also increasing inhibition of excitatory pyramidal neurons (Alyea et al. 2008, Russell et al. 2012, Bodo et al. 2006, Tan et al. 2012). In addition, ERbeta activation inhibits estradiol mediated synaptogenesis (Szymczak 2006). Together with age-related changes in ERalpha expression, interaction with ERbeta may underlie the increasing inability of estradiol to promote dendritic growth in aged rats (Adams et al. 2001, Miranda et al. 1999). In the hippocampus, there is a non-monotonic response curve following estradiol administration, which shifts during aging, with large amounts of estradiol needed to improve memory in older rats, enough to impair function young animals,. This shift appears to be due to changes in the relative expression of ERalpha and ERbeta, which could explain the increased levels of estradiol that are sometimes needed to see a similar cognitive facilitation in older rats as that seen in young rats (Foster et al. 2012). The cortex also shows age related changes in the ratio of ERalpha to ERbeta expression. Levels of ERalpha were 50% higher in 24 m.o. male mice compared to 3 m.o., while ERbeta did not change (Arimoto et al. 2013). Estradiol-regulated neurite outgrowth also decreased in aged animals, but was partially restored by increasing ERalpha expression. This is in contrast to female mice, where ERalpha in the cortex did not change, but ERbeta declined (Sharma et al. 2006). Together, these studies underscore not only the importance of interactions between ERalpha and ERbeta, but they also underscore the need to not generalize results of studies in females to males.

1.3 INTRACELLULAR REGULATION BY ESTRADIOL

ERalpha and ERbeta have traditionally been seen as primarily nuclear receptors that, after being collected with other proteins and transcription factors, bind estrogen response elements (ERE) to regulate transcription of a wide variety of genes. More recently, it has been seen that estradiol can also rapid effects on cell function and behavior (Srivastava and Perez 2011, Srivistava et al. 2008, Micevych et al. 2009, Taziaux 2007). Acute doses of estradiol facilitate sexual behavior in rats and quail in less than half an hour (Charlier et al. 2010, Thakur and Sharma 2007), and Cross and Roselli (1999) found that estradiol facilitated genital sniffing and mounting in a dose dependent within 35 minutes of being adminitrated. Acute effects of estradiol have also been observed in tests of spatial memory tasks. Estradiol might also influence behavior by interacting with receptor types other than ER, such as NMDA, mGluR1 and K+ ion channels (Bonder et al. 2009, Druzin et al. 2011, de Anglemont de Tassigny et al. 2007, Dewing et al. 2007). Finally, one other possible mechanism for modulation is evidenced in the hippocampus, where estradiol acts rapidly to regulate synaptogenesis in both males and females (Hu et al. 2008, Prange-Kiel and Rune 2006, Foy et al. 2010).

Although hormone receptor expression in the POA continues to be normally regulated in 12 month old rats, these animals will still display behavioral impairments (Wu et al. 2009). This indicates a change in receptor function. One possible mechanism might be a change in hormone binding properties. As evidence of this, the number of ERalpha binding sites in rats was lower at 30 months than 3 months. This change in hormone binding properties might lead to decreases in behavioral responsiveness to

hormones, such as those seen in older animals (Haji et al. 1981). In addition, several co-regulators and transcription factors needed for ERalpha to bind ERE sites, such as SRC-1, may also undergo age-related changes. This makes is possible for estradiol's regulation of transcription to change with age, even if the receptor number does not change (Thakur and Kumar 2007, Thakur and Sharma 2007). It is also possible for changes in co-regulators and transcription factors to be region and sex specific. This is true as in the cortex (Cx), where aged mice (65 weeks old) had decreased binding of core promoters compared to young animals (25 weeks) (Thakur and Kumar 2007). In the cortex of 18 month old female rats genes specifically associated with rapid estradiol signalling showed increased expression in response to estradiol, compared to 4 month old females. This suggests a disparity in basal pathway activity in young vs. aged animals (Aenlle 2010). Changes in regulation by estradiol in males could also be specific to certain pathways, but unfortunately similar experiments have not been performed in males, and not enough information exists at this point to provide a more detailed picture (Aenlle 2010).

The MPOA integrates sensory input with autonomic output, coordinating stimuli in the environment with the appropriate behavioral response (Hull and Dominguez 2007). Lesions of the MPOA produce greater impairments of sexual behavior than do those to the MeA or BnST (Hull and Dominguez 2006). Fos, the protein byproduct of the immediate early gene c-Fos, is increased in the MPOA of sexually experienced males, and in response to copulation (Hull and Dominguez, 2007). Different subsets of preoptic cells show Fos activation in response to specific copulatory behaviors; there is increased

activation in males that ejaculate several times compared to those that ejaculate only once, or only mount the female (Coolen et al 1997, Balthazart and Ball 2007), indicating a positive correlation between levels of sexual activity and activation of the cells in the MPOA.

1.4 Behavioral Regulation by Estradiol

In the end, changes in expression and enzymatic activity must be linked to a behavioral response to be functionally significant. Sexual behavior has been one of the most thoroughly and widely studied behaviors that are regulated by gonadal steroid hormones, and shows substantial age-related impairments (Chamber and Phoenix, 1984). Although some amount of androgenic activity, either by testosterone or DHT, is needed to see a complete behavioral repertoire, estradiol is still very important for sexual activity. DHT alone, or testosterone given with an aromatase inhibitor, do not result in significant recovery of behavior in castrates (Attila 2009, McGinnis and Dreifuss 1989, Roselli et al. 2003). However, if DHT is given with estradiol or an ERalpha agonist, a reinstatement of behavior is seen in young castrated animals (Russell et al. 2012). Though estradiol replacement still facilitates sexual behavior in 24 m.o. males compared to aged castrates, this recovery of behavior does not extend to erasing age-related deficits; even with hormone replacement to levels equal to young males, old males still show behavioral impairments (Chambers and Phoenix, 1986). 12 m.o. intact rats given testosterone, or castrates given testosterone, also failed to demonstrate a full range of sexual behaviors, although similar to the animals in the previous study mature castrates could perform up to levels seen before castration (Wu and Gore, 2010, Chambers and

Phoenix ,1984), which suggests that testosterone's function is more important than its concentration alone.

Many regions in the brain are activated in response to sexual behavior. The functions of these areas include mediating chemosensory and somatosensory information, as well as autonomic responses, such as erections and ejaculation, in order to achieve successful copulation (review in Hull and Dominguez 2006). The olfactory bulbs (OB), medial amygdala (MeA) and bed nucleus of the stria terminalis (BnST) are all steroid sensitive areas involved in processing sexually relevant olfactory cues. The MeA and BnST both project to the medial preoptic area (MPOA), a region essential for the expression of sexual behavior.

The MPOA integrates sensory input with autonomic output, coordinating stimuli in the environment with the appropriate behavioral response (Hull and Dominguez 2007). Lesions of the MPOA produce greater impairments of sexual behavior than do those to the MeA or BnST (Hull and Dominguez 2006). Fos, the protein byproduct of the immediate early gene c-Fos, is increased in the MPOA of sexually experienced males, and in response to copulation (Hull and Dominguez, 2007). Different subsets of preoptic cells show Fos activation in response to specific copulatory behaviors; there is increased activation in males that ejaculate several times compared to those that ejaculate only once, or only mount the female (Coolen et al 1997, Balthazart and Ball 2007), indicating a positive correlation between levels of sexual activity and activation of the cells in the MPOA.

1.5 Neurotransmitters and Sexual Behavior in the MPOA

Within the MPOA, testosterone, glutamate, and doamine all play important roles in the control of sexual behavior. Glutamate in the MPOA increases during copulation, possibly due to stimulation from glutamatergic neurons in the MeA, peaking around ejaculation before falling back to baseline (Dominguez et al. 2006, Hull and Dominguez 2006). Reverse dialysis of glutamate in to the MPOA increased dopamine concentrations, as did stimulation of the MeA or exposure to a female (Hull et al. 1995, Dominguez et al. 2001, Dominguez et al. 2004). Dopamine release in the MPOA is necessary for copulation in males (Dominguez and Hull, 2005). Microinjections of dopamine, or dopamine agonists, facilitate sexual behavior, while dopamine antagonists impair both sexual motivation and

copulation (Dominguez and Hull, 2005, Hull and Dominguez 2007, Wersinger and Rissman 2000).

Castration reduces basal extracellular DA, and eliminates female-stimulated dopamine release (Hull *et al.* 1997, Phillips-Farfan *et al.* 2008, Putnam *et al.* 2003). Testosterone replacement restores copulatory behavior, basal dopamine and female-stimulated release (Wood 1996, McGinnis and Dreifuss 1989, Putnam et al. 2003), while castrates given DHT alone failed to show either increases in basal extracellular or female-stimulated release compared to castrates, while estradiol alone increased basal dopamine concentrations, but was insufficient to produce female-stimulated dopamine (Putnam et al. 2005). Dopamine levels also decline with age, yet in middle aged animals basal dopamine release in the MPOA declines only in animals that no longer ejaculated; animals that successfully mounted, intromited, and ejaculated had DA levels similar to those measured in younger animals (Chen *et al.* 2007, Sato *et al.* 1998).

Glutamate increases dopamine release during copulation by increasing the production of nitric oxide (NO). Glutamate binds NMDA receptors, increasing intracellular Ca^{2+} and activating NOS (Dominguez and Hull, 2005, Garthwaite and Boulton, 1995). NOS inhibitors prevent female stimulated dopamine release, while reverse dialysis of the NO precursor L-arginine into the MPOA increased basal dopamine levels (Lorrain et al. 1996, Dominguez and Hull 2005). Estradiol contributes to NO production both by increasing NOS expression, and by promoting NMDA/NOS coupling and NOS activation (Putnam et al. 2005, d'Anglemont de Tassigny et al. 2009).

1.6 SUMMARY

Together with the studies on hormone receptor expression, these findings suggest that aged animals retain the ability to respond to DA and bind estradiol. This also suggests and that animals failing to copulate might have deregulated interactions between testosterone metabolites and DA, which may explain why increased steroid hormones do not reinstate copulation. Given the importance of estradiol in mediating DA release, we hypothesized that age-related estrogenic changes attenuate dopamine activity and thus impair behavior. The examine this we looked at

1. Activation of ER and AR containing cells in the MPOA of mature and aged males after copulation
2. Expression of target genes in mature and aged males after estradiol administration in nuclei involved in regulating sexual behavior, specifically the MPOA, the MePD and the BnST.
3. Changes in basal and female-stimulated dopamine release in the POA of mature and aged castrated males given vehicle or estradiol replacement.

Chapter 2: Sexual Experience Influences Mating-Induced Activity in Nitric Oxide Synthase-containing Neurons in the Medial Preoptic Area

Nutsch, V. L., Will, R. G., Hattori, T., Tobiansky, D. J., & Dominguez, J. M. (2014). Sexual experience influences mating-induced activity in nitric oxide synthase-containing neurons in the medial preoptic area. Neuroscience Letters, 579, 92–96. http://doi.org/10.1016/j.neulet.2014.07.021.

I worked with Dr. Dominguez to design the study, I was in charge of running the study and analyzing the data, and Dr. Dominguez and I wrote the paper together.

2.1 Abstract

Nitric oxide (NO) acts in the medial preoptic area (mPOA) of the hypothalamus to facilitate the expression of male sexual behavior and has also been widely implicated in mechanisms of experience, learning, and memory. Using immunohistochemistry for Fos, as a marker for neural activity, and nitric oxide synthase (NOS), the enzyme that catalyzes the production of nitric oxide (NO), we examined whether sexual activity and sexual experience influence Fos co-expression in NOS-containing neurons in the mPOA of male rats. Consistent with previous findings, results indicate that mating increased activity in the mPOA, and that sexual experience facilitated the expression of sexual behaviors, together with increased mating induced Fos and NOS in the mPOA. Results also indicate that mating increased co-expression of Fos in NOS-containing neurons, and that this increase was highest in animals undergoing their first sexual encounter,

indicating that initial sexual experience increases NO production in the mPOA of male rats.

2.2 INTRODUCTION

Nitric oxide (NO) acts in the peripheral and central nervous system (CNS) to influence the expression of sexual behavior in males. Studies show that peripheral administration of the nitric oxide synthase (NOS) inhibitor l-nitro-arginine methyl ester (l-NAME) impairs sexual behavior, as animals receiving drug displayed fewer intromissions and ejaculations, required more time to reach intromissions and ejaculations, and a larger number of them failed to ejaculate, when compared to those receiving vehicle administration (Benelli et al. 1995, Hull et al. 1994). By contrast, the NO donor l-arginine facilitated sexual behavior, as evidenced by shorter latencies to intromit and ejaculate, a greater number of ex copula erections, and an increased number of animals copulating after drug administration (Hull et al. 1994). Together these findings suggest that NO influences male sexual behaviors. NO acts in several brain regions to modulate the expression of male sexual behavior. One region in particular, the medial preoptic area (mPOA), plays a central role in the regulation of copulation in all studied species, and NO works in this region to facilitate mating (Hull and Dominguez, 2007). The significance of the mPOA for mating has been widely demonstrated; for example in rats, lesions of the mPOA eliminated mounts, intromissions, and ejaculations in 71%, 86%, and 100% of the animals, respectively, whereas all control animals displayed normal behavior (Arendash et al. 1983) (reviewed in Hull and Dominguez, 2007). Conversely, stimulation of the mPOA facilitates mating, as evidenced by a reduction in the number of mounts and intromissions preceding

ejaculation, as well as reduced mount latencies and post ejaculatory intervals following stimulation (Malsbury 1971, reviewed in Hull and Dominguez, 2007). The act of mating itself also activates the mPOA, as evidenced by an increase in the number of Fos-positive cells, used as a measure of neural activity (Pfaus and Heeb 1997), which is highest in sexually experienced animals (Lumley and Hull 1999). Finally, the mPOA receives indirect input from all sensory modalities, and in turn projects to premotor areas responsible for sexual response, further supporting its central role in the regulation of mating. In view of a significant role for the mPOA and NO in male sexual behavior, it is not surprising that NO acts in the mPOA to facilitate mating. This is supported by studies showing that microinjections of l-NAME or the NO precursor l-arginine directly into the mPOA inhibited or facilitated sexual behavior, respectively, much in the same way as intraperitoneal injections (Lagoda et al. 2004). This suggests that behavioral changes occurring after systemic drug administration were due at least in part to influences of drug directly in the mPOA. Itis interesting to note that the behavioral effects of l-NAME vary as a function of the animal's experience. Namely, sexually experienced animals showed less impairment than those who were naïve (Lagoda et al. 2004), again suggesting that NO mediates experience-induced changes in the mPOA and ensuing behaviors. The idea that NO influences sexual learning is consistent with other studies, which present a role for NO in the regulation of experience, memory, and learning (Susswein et al. 2004). Consider for example that l-NAME injected into the olfactory bulb of ewes prevented learned recognition of their offspring (Kendrick et al. 1997), whereas in rats, it impaired performance on both spatial and object recognition tasks (Bohme et al. 1993). NO activates

the soluble form of guanylyl cyclase (sGC), which then activates cGMP. Administration of cGMP inhibitors has been shown to impair the formation of olfactory memories and the consolidation of fear memories (Bhat et al. 1996,Ota et al. 2010), a further demonstration of memory modulation by NO. It is still not clear, however, whether NO acts in the mPOA to mediate behavioral changes that accompany sexual experience. One study suggests such a role, as blocking NOS also inhibited experience-induced sexual improvements in rats (Lagoda et al. 2004). Given the importance of the mPOA for sexual behavior, the role of NO in experience-induced neuronal changes, and evidence that experience influences NO in the mPOA, we hypothesized that sexual experience would influence mating-induced stimulation of NOS-containing neurons in the mPOA of male rats. To test this hypothesis, we quantified NOS-positive, Fos-positive, and co-localized cells in the mPOA of male rats and compared the number of labeled cells as a function of sexual activity and sexual experience.

2.3 GENERAL METHODS

2.3.1 Subjects

Eighty-four Long–Evans male rats (Harlan, Indianapolis, IN; 90 days old at arrival) were housed individually in large plastic cages, in a climate-controlled room, on a 14:10 h light–dark cycle, with lights off at 10:00 a.m. and on at 8:00 p.m. Food and water were freely available. Conspecific females (n = 21) were ovariectomized under ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (4 mg/kg) anesthesia. They were broughtinto behavioral estrus with 4 g estradiol benzoate (s.c.) 48 h before, and 400 g (s.c.) progesterone 4 h before testing. Behavioral receptivity was confirmed by

placing the female with a stud male shortly before the test began. All procedures were done in accordance with the National Institutes of Health Guidelines for the Use of Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Subject-male rats were randomly assigned to one of the following four conditions, with 21 animals per group: animals who were sexually experienced, but did not mate on the day of sacrifice (experienced but no sex, Exp-NoSex); animals who were sexually experienced and also mated on the day of sacrifice (experienced and sex, Exp-Sex); animals who were sexually naïve and did not mate on the day of sacrifice (inexperienced and no sex, Inexp-NoSex); animals who were sexually naïve but experienced mating for the first time on the day of sacrifice (inexperienced and sex, Inexp-Sex). Sexual experience consisted of mating with a sexually receptive female for 90 min, every other day, for 6 days before the day of sacrifice, for a total of 9 h. On the 6th day, animals were observed to confirm that they achieved at least two ejaculations during the final experience session. Two animals that did not meet this criterion were excluded from further testing. Two days separated the last experience day and the test day, when animals were sacrificed. Behavior data analyzed and reported in Table 1 were obtained on the test day, which followed 2-days after the final experience sessions. Animals in the mated groups were allowed to copulate to one ejaculation. Animals that failed to copulate after 1 h were removed and excluded from further analysis. No-sex controls were handled, but females were not introduced into their home cage. This approach allowed us to distinguish between mating-induced and experience-induced

changes in brain activity. All animals were sacrificed with an overdose of sodium pentobarbital (100 mg/kg), 1 h after ejaculation or the end of testing..

2.3.2 Immunohistochemistry

Under pentobarbital anesthesia, rats were perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH = 7.35). Brains were removed, post fixed for 1 h in the same fixative at room temperature, and stored in 30% sucrose at 4 ◦C. Coronal sections were cut at 35 m and stored in cryoprotectant solution. Sections containing the mPOA underwent immunohistochemical staining for neuronal NOS and Fos. Washes in PB, 4 times for 5 min, preceded all incubations. Sections underwent the following incubations: 1% H2O2 in PB, and then blocked in 2% normal goat serum and 0.1% Triton X-100 (blocking solution); rabbit anti-nNOS primary antibody and blocking solution, overnight at room temperature (1:12,000; Immunostar, Hudson, WI). The following day, sections were incubated in anti-rabbit biotinylated secondary antibody (1:500 in blocking solution; Vector Labs, Burlingame, CA) before avidin–biotin conjugate (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized with a diaminobenzidine (DAB)–nickel chromogen solution (Sigma, St. Louis, MO) to yield a purple-black precipitate, incubation lasted 10 min. After washing thoroughly with PB, sections were incubated with mouse anti-Fos primary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in blocking solution. Anti-mouse biotinylated secondary antibody (1:500 in blocking solution; Vector Labs, Burlingame, CA) preceded incubation with the avidin-biotin conjugate, and was visualized with a DAB chromogen solution without nickel yielding a brown precipitate.

Sections were dehydrated, mounted, and coverslipped with DPX (VWR, Radnor, PA). For negative controls, sections underwent the same immunostaining procedure, except the nNOS, Fos, or both primary antibodies were excluded. When introducing and removing tissue from incubations, experimenters were careful to minimize the transfer time separating the first and last set of tissue, the transfer time averaged 40 s for all incubations including DAB. Light microscopy was used to quantify the number of cells containing nNOS-ir, Fos-ir, and double-labeled cells. The mPOA was examined bilaterally and labeled cells were counted in a 300 × 400 m area in the medial preoptic nucleus (MPN), a central nucleus in the mPOA. Counts were performed manually using ImageJ. Six slices were counted bilaterally (two each rostral, medial, and caudal).

2.3.3 Data Analysis

A two-way analysis of variance (ANOVA; mating x experience) was performed to probe for differences in the number of immunopositive cells. Bonferroni correct one-way ANOVAs were used to probe for significant differences among individual means, when interactions were present. Also, Welch two sample t-tests were used to probe for differences in behavioral measures. Data analyses were performed with R (version 2.15.1).

2.4 RESULTS

Consistent with previous findings, experienced animals had shorter latencies to mount ($t(12.03) = 2.234$, $p < 0.05$), intromit ($t(16.03) = -3.014$, $p < 0.01$), and ejaculate ($t(24.73) = 3.800$, $p < 0.001$)(Fig 2.1). Sexual experience did not influence mount or intromission frequency. The number of cells containing NOS changed as a function of experience, with sexually experienced animals having a greater number of NOS-positive cells (Fig. 2.1). When grouped together, statistical analysis revealed main effects of experience ($F(1,19) = 16.3031$, $p < 0.001$) and sex ($F(1,19) = 11.761$, $p < 0.001$), but no interactions; for this reason, post-hoc analyses were not performed to assess individual group differences. Also consistent with previous findings, mating increased the number of Fos-positive cells in the mPOA (Fig. 2.1).

Finally, mating increased activity in NOS-containing neurons. The percentage of NOS-containing neurons that co-expressed Fos with mating was highest in previously inexperienced animals undergoing their first sexual encounter, when compared to all other groups. Analysis of the percent of NOS-Fos double-labeled cells revealed no main effect of experience ($F(1,19) = 0.030$, $p > 0.05$), but there was a main effect for sex ($F(1,19) = 88.030$, $p < 0.001$) and a sex by experience interaction ($F(1,19) = 14.372$, $p < 0.001$) (Fig. 2.2). Bonferroni correct one way ANOVAs revealed that colocalization of NOS and Fos was highest in animals that had sex on the day they were sacrificed ($p < 0.001$). Within the groups that had sex on the day of sacrifice, the greatest colocalization was observed in previously inexperienced animals that mated for the first time on the day

of sacrifice ($p < 0.001$). There were no significant differences between animals not having sex the day of sacrifice (Exp-No Sex vs. Naïve-No Sex).

In order to verify that changes in activation were specific to the mPOA, the number of immuno-reactive cells for Fos and NOS was also examined in the ventrolateral preoptic area (VLPO), a region found at the same anteroposterior level as the mPOA. Analyses of the VLPO revealed no significant differences in the number of NOS-ir, Fos-ir, or colocalized cells between any of the four groups. Specifically, the number of NOS-positive cells in the VLPO was as follows for each group (mean ± SEM): Exp-No Sex, 39 ± 2.47; Exp-Sex, 34.8 ± 4.06; Naïve-No Sex, 43.6 ± 4.6; Naïve-Sex, 33.5 ± 3.84. The number of Fos-positive cells in the VLPO was as follows for each group (mean ± SEM): Exp-No Sex, 8.0 ± 1.24; Exp-Sex, 10.0 ± 0.68; Naïve-No Sex, 7.9 ± 0.78; Naïve-Sex, 9.0 ± 0.82. The number of cells containing both NOS and Fos in the VLPO was as follows for each group (mean ± SEM): Exp-No Sex, 2.2 ± 1.24; Exp-Sex, 3.0 ± 0.68; Naïve-No Sex, 2.0 ± 0.78; Naïve-Sex, 2.5 ± 0.82.

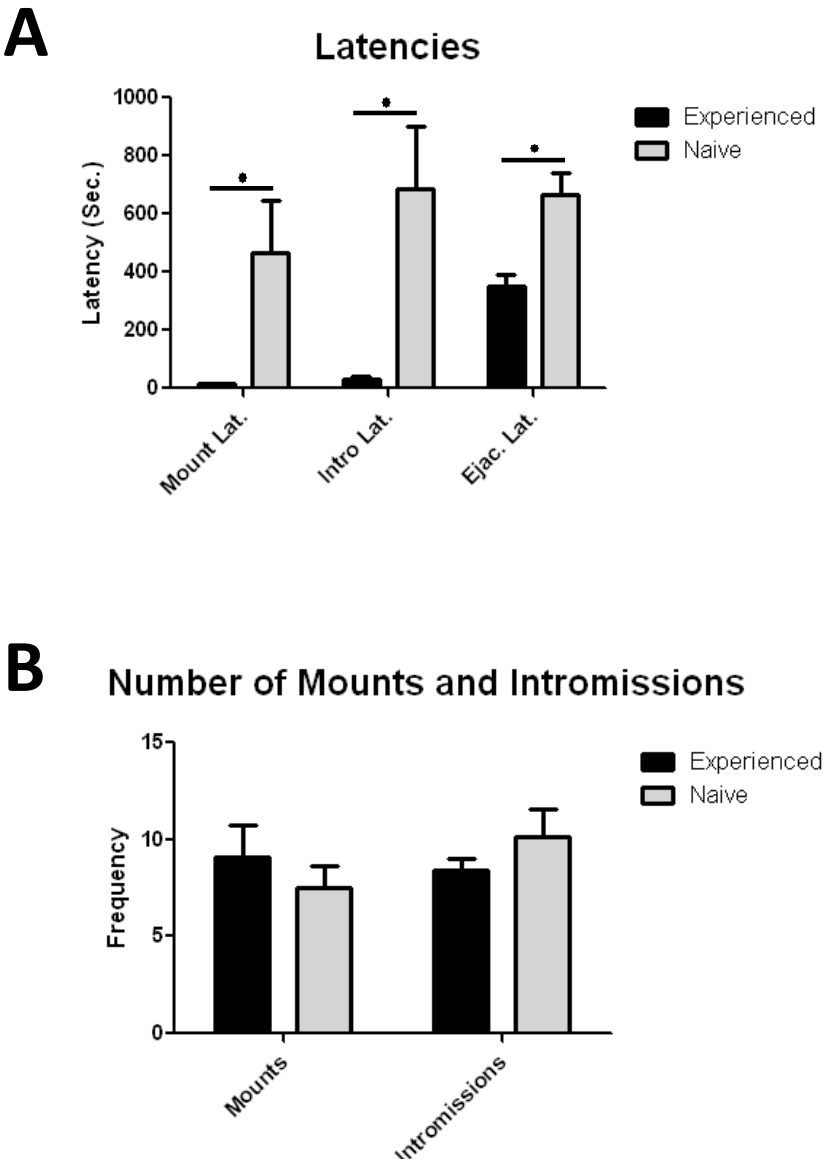

Figure 2.1 Sexual Behavior in Experienced and Naïve Males

Sexually experienced males had decreased Mount, Intromission and Ejaculation latencies compared to naïve animals (A), but no difference in the number of mounts or intromissions. Values are expressed as mean ± SEM, ** $p < 0.001$.

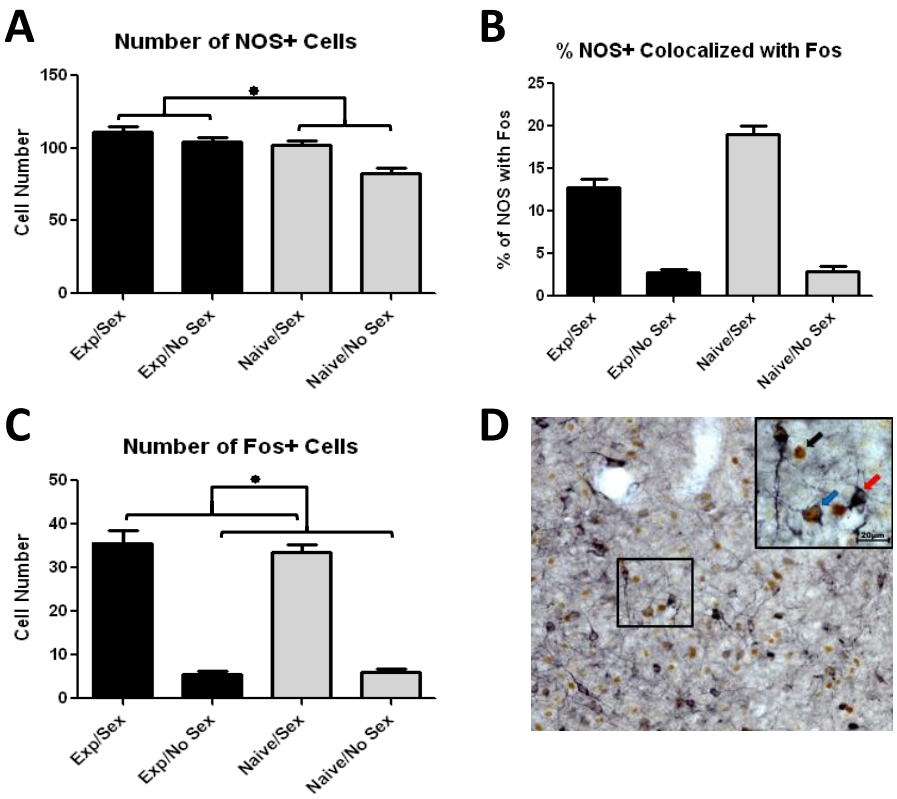

Figure 2.2 NOS and Fos in the MPOA

Sexual experience and sexual activity increased NOS (A), while sexual activity increased Fos in the mPOA (C). Sexual activity also increased activation of NOS-positive cells; this increase was highest in previously naive males during their first sexual encounter (C). A sample micrograph obtained in the medial preoptic nucleus (central region in the mPOA) of a sexually experienced male who mated on the day of testing (D) shows in the upper right insert, a black arrow pointing to a Fos-only cell, the red arrow pointing to a NOS-only cell, and the blue arrow pointing to a co-localized cell, scale bar is 20 m. Values are expressed as mean ± SEM, ** $p < 0.001$.

2.5 DISCUSSION

The present experiment shows that mating increases Fos in the mPOA of male rats, these results are consistent with previous findings (Hull and Dominguez, 2007). However, we are the first to report that mating stimulates NOS-containing neurons and that this stimulation is significantly higher in the mPOA of previously inexperienced males undergoing their first sexual encounter. This finding is noteworthy because sexually experienced animals display increased sexual efficiency, as evidenced by an increase in ejaculation frequency and decreased latencies, when compared to inexperienced males (Hull and Dominguez, 2007). This change in behavior might result from plasticity in a sex-relevant brain region like the mPOA. Given that NO modulates synaptic function and plasticity (Dawson et al. 1998, Hawkins et al. 1998, Schuman et al. 1994, Susswein et al. 2004), we speculate that increases in NO during an initial sexual encounter potentiate responsiveness of cells in the mPOA, thus easing the expression of subsequent behaviors in experienced males.

2.5.1 Nitric Oxide and Male Sexual Behavior

The importance of NO in the mPOA for mating has been previously established. Studies show that mating stimulates NOS containing neurons in the mPOA of gerbils (Simmons and Yahr, 2011), administration of l-NAME impairs intromissions and ejaculations, and l-arginine facilitates mounts, intromissions and ejaculations in rats (Hull et al. 1994). While we did not directly measure experience-induced changes in NO production, we discovered greater activation of NOS neurons in animals during initial experience, leading to the inference that NO modulates synaptic function in the mPOA,

thereby facilitating the responsiveness of these neurons to sexual stimuli in experienced males. This experience-induced change in neuronal function may be one factor that is responsible for increased sexual efficiency seen in experienced males. In line with this conclusion, l-NAME microinjected into the mPOA before exposure to an estrous female prevents the experience-induced facilitation of mating; however, when given to experienced rats l-NAME did not disrupt behavior as severely as in inexperienced males (Lagoda et al. 2004), suggesting, as do our findings, that increasing NO during a first sexual encounter is important and changes the mPOA to ease the expression of behaviors that are associated with experience.

2.5.2 Nitric Oxide in Learning and Memory

While we did not directly measure memory or plasticity in our experiments, we should like to speculate on the possible mechanism mediated by our findings. Since its discovery as an intercellular neuromodulator (Bredt et al. 1992, Garthwaite et al. 1988), mediation of synaptic plasticity and neurotransmitter release are the two major functions ascribed to NO (Bredt et al. 1992). Soluble guanylyl cyclase (sGC), which activates cGMP, is the most common target of NO. The resultant increase in cGMP affects ionic channels, protein kinases, phosphodiesterases, and ribosyl cyclase (Dawson et al. 1998). Some effects of NO are also independent of sGC and may be more closely linked to NMDA receptor stimulation. One such example is activation of p21ras, which is displayed in the adult and developing nervous system, and plays an important role in mediating growth factors, neuronal survival, differentiation, long-lasting neuronal responses and other forms of plasticity (Yun et al. 1998). With regards to modulation of

transmitter release, NO induces calcium-independent vesicular release of transmitters from synaptic terminals (Yun et al. 1998), and has been shown to inhibit dopamine and glutamate transporter systems, thereby prolonging the synaptic presence of these transmitters (Pogun et al. 1994). NO also modulates the placement of proteins associated with vesicular release at the synapse, and increases spontaneous EPSPs in cultured neurons, this being another possible mechanism through which NO increases transmitter release (Feil and Kleppisch, 2008). Because cGMP is implicated in the localization of synaptic spines, NO might also be acting via cGMP to ensure proper alignment of presynaptic terminals with postsynaptic receptors (Feil and Kleppisch, 2008). Combined, these findings support an important role for NO in transmitter release and synaptic plasticity, which might be the same mechanism via which it changes synaptic function in the mPOA during sexual experience.

2.5.3 Nitric Oxide, Glutamate and Dopamine

Perhaps more precisely, NO could potentiate glutamate and dopamine function in the mPOA, which are known to promote sexual behavior in males. Supporting this idea, dopamine agonists microinjected into the mPOA facilitate mating, whereas antagonists impaired copulation, genital reflexes, and sexual motivation (Dominguez and Hull, 2005). Moreover, extracellular dopamine increases during mating in male rats (Dominguez and Hull, 2005). Similarly, glutamate in them POA also promotes copulation. In support of this conclusion, microdialysis experiments showed that levels of glutamate increased in the mPOA during mating and then decreased following ejaculation, concurrent administration of glutamate agonists into the mPOA in turn

increased glutamatergic stimulation and also facilitated behaviors (Dominguez, 2009). It appears that glutamatergic stimulation of NMDA receptors is partly responsible for glutamate's behavioral effects, as evidenced by histological analysis of the mPOA showing that nearly all cells containing mating-induced Fos also contained NMDA receptors and, furthermore, that mating phosphorylated NMDA receptors in the mPOA of male rats (Dominguez, 2009). Finally, microinjections of the NMDA receptor antagonist dizocilpine (MK-801) significantly impaired sexual behavior in male rats (Dominguez, 2009).

Several other studies support the conclusion that NO promotes mating by acting on glutamate and dopamine function in the mPOA. Consider for example that reverse dialysis of the NO precursor l-arginine through a microdialysis probe in the mPOA increased basal dopamine levels, whereas reverse dialysis of l-NAME, the antagonist, inhibited female-stimulated dopamine release and sexual activity (Dominguez and Hull, 2005). NO also mediates glutamate-stimulated dopamine release, as reverse dialysis of l-NAME, but not its inactive isomer d-NAME, decreased both basal levels and female-stimulated dopamine release in the mPOA (Dominguez and Hull, 2005). We should note that our results showed increase mating-induced Fos in the mPOA, however, we did not isolate possible influences of olfaction from influences of mating itself. Nevertheless, given the large number of studies showing mating-induced Fos in the mPOA, we are confident that this increase is due at least in part to mating.

2.5.4 Conclusion

In conclusion, mating stimulated NOS-containing neurons in the mPOA of male rats, which suggests increased production of NO. This stimulation was highest in previously inexperienced animals during their first sexual encounter. This suggests that NO in the mPOA might play some role in the plasticity that accompanies sexual learning. Because increases in NO are known to change synaptic function, we postulate that NO in the mPOA increases during an initial sexual encounter, this then potentiates cells in this region to respond more readily in the presence of subsequent sexually exciting stimuli.

Chapter 3: Reproductive Aging in Male Rats: Androgen Receptor and Estrogen Receptor Alpha in the Medial Preoptic Area

3.1 Abstract

Testosterone is the main circulating steroid hormone in males, and acts to facilitate sexual behavior via both reduction to dihydrotestosterone (DHT) and aromatization to estradiol. The mPOA is a key site involved in mediating actions of androgens and estrogens in the control of masculine sexual behavior, but the respective roles of these hormones is not fully understood. As males age they show impairments in sexual function, and a decreased facilitation of behavior by steroid hormones compared to younger animals. We hypothesized that an anatomical substrate for these behavioral changes is a decline in expression and/or activation of hormone receptor-sensitive cells in the mPOA. We tested this by quantifying and comparing numbers of AR- and ERα-containing cells, and Fos as a marker of activated neurons, in the mPOA of mature (4-5 months) and aged (12-13 months) male rats, assessed one hour after copulation to one ejaculation. Numbers of AR- and ERα cells did not change with age or after sex, but the percentage of AR- and ERα-cells that co-expressed Fos were significantly up-regulated by sex, independent of age. Age effects were found for the percentage of Fos cells that co-expressed ERα (up-regulated in the central mPOA) and the percentage of Fos cells co-expressing AR in the posterior mPOA. Interestingly, serum estradiol concentrations positively correlated with intromission latency in aged but not mature animals. These data show that the aging male brain continues to have high expression and activation of both AR and ERα in the mPOA after copulation, raises the possibility that differences in

relationships between hormones, behavior, and neural activation may underlie some age-related impairments.

3.2 INTRODUCTION

Sexual behavior in many species of male mammals undergoes marked declines during aging for both motivational and copulatory behaviors, starting in middle age and becoming more severe with more advanced aging (Amstislavskaia et al. 2010, Smith 1992). These behaviors are highly dependent on appropriate secretion patterns and concentrations of steroid hormones, especially androgens and estrogens. Castrated males exhibit impaired copulatory behavior, but will exhibit behavior similar to that of intact animals if give testosterone replacement (McGinnis and Dreifuss, 1989 Park *et al.* 2007). Both the androgenic and estrogenic metabolites of testosterone are required for the full manifestation of these behaviors. Castrated males administered hormone replacement using the non-aromatizable androgen 5α-dihydrotestosterone (DHT) alone, or those given testosterone with an aromatase inhibitor, still show behavioral impairments, underscoring the importance of estradiol for copulation (Hull and Dominguez 2006, Putnam et al. 2003). In fact, administration of estradiol to castrated males restores certain components of the sexual behavior repertory, including both motivational behaviors, such as anticipatory levels changes and mounting, and consummatory behaviors, such as intromissions (Attila et al. 2009, Roselli et al 2003). During aging, serum testosterone concentrations decline, but interestingly, this does not correlate with declines in sexual behavior (Smith 1992, Wu and Gore 2009, Chambers and Phoenix 1984, Chambers and Phoenix 1986). Regarding estradiol, replacement of this hormone to aging male rats does

not fully restore copulatory measures to those seen young animals (Chambers and Phoenix 1986). Thus, both classes of hormones are needed, but their exact roles still require elucidation.

In the neural network of brain nuclei that underlies sexual behavior in males, the medial preoptic area (mPOA) plays a key role in both sexual motivation and copulatory performance (Yeh et al. 2009, Hull and Dominguez 2007). Lesions to the mPOA impair, while electrical stimulation facilitates, male sexual behavior (Liu et al 1997, Rodriguez-Manzo 2000). The mPOA is a site of hormone action in the control of sexual behavior (Russell 2012, Wood and Williams 2001), with a high density of steroid hormone receptors, including estrogen receptor alpha (ERα) and androgen receptor (AR) (Ottinger *et al.* 1995, Perez et al. 2003, Wu et al. 2009, Wu and Gore 2010, Simerly et al 1990). These ERα and AR sensitive cells in the mPOA are both activated by copulation (Greco 1998).

Despite this knowledge about the importance of the mPOA, testosterone and estradiol in male sexual behavior, relatively little is known about this interplay during reproductive aging. We hypothesize that age-related impairments in sexual behavior are due at least in part to impairments in the hormone responsiveness of the mPOA through age-related changes in both the expression and activation of the AR and ERα in this region. To test this, we quantified ERα-positive, AR-positive and Fos-positive cells in the mPOA of mature and aged rats. This work was conducted in the framework of both age and prior sexual experience, the latter exerting a strong influence on sexual behavioral outcomes (Hull and Dominguez 2006; Wu and Gore 2010, Lisk and Heimann 1980).

3.3 MATERIALS AND METHODS

3.3.1 Animals and Husbandry

Sprague-Dawley male rats (Harlan, Indianapolis, IN; 3 months (n = 24) or 12 months (n=13) at arrival) were pair housed in large plastic cages, in a climate-controlled room, on a 14:10 h light/dark cycle, with lights off at 10:00H and off at 20:00H. Food and water were freely available. Conspecific females (n = 16) were purchased as young adults, and ovariectomized under ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (4 mg/kg) anesthesia. They were brought into behavioral estrus with an injection of 4 μg estradiol benzoate (s.c.), followed 44 hours later by an injection of 400 μg (s.c.) progesterone. Testing took place 4 hours later. Sexual receptivity of females was confirmed by placing her into a cage with a separate stud male shortly before the test began and watching for lordosis. All procedures were done in accordance with the National Institutes of Health's Guidelines for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

3.3.2 Behavioral Testing

Males were used at two ages: mature adult (MAT) and aged (AG). To match sexual experience, MAT males (3 months) were allowed to mate with a sexually receptive female for 90 min, every other day, for 14 days, for a total of 7 experience sessions. On an 8th day animals were observed to confirm that they achieved at least two ejaculations during the final experience session. Two males failed and were excluded

from further testing. Aged males (12 months) were retired breeders at purchase and were not given further experience sessions in the lab.

Sexual behavioral data were obtained on the test day, which took place at least 2 days after the last sexual behavioral experience for the MAT males. Animals of both ages in the sex groups were allowed to copulate to one ejaculation. One MAT animal failed to copulate after 1h and was excluded from further analysis. A no-sex control group of each age was handled, but females were not introduced into their home cage. This resulted in four groups: MAT males (approximately 4 months at euthanasia) given sex (MAT-S, n=16), MAT males with no sex (MAT-NS, n=5), and the AG counterparts (approximately 13 months at euthanasia) with (AG-S, n=8) or without (AG-NS, n=5) copulation. We note that while the n's of the AG groups is small due to our difficulty in attaining animals at the appropriate age, the study was adequately powered. All animals were euthanized with an overdose of sodium pentobarbital (100 mg/kg), 1 hour after ejaculation (sex groups) or handling (no-sex groups).

3.3.3 Immunohistochemistry

Rats were perfused transcardially with saline under pentobarbital anesthesia, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (0.1M PB; pH = 7.35). Brains were removed, postfixed for 1h in the same fixative at room temperature, and stored in 30% sucrose at 4°C. Coronal sections were cut at 35 μm into four equal series through the mPOA and stored in cryoprotectant (30% ethylene glycol, 30% sucrose, 0.00002% sodium azide in 0.1M PB) at -20°C until use.

Sections underwent immunohistochemical staining for either ERα or AR, with each nuclear receptor double-labeled with Fos, the immediate early gene product and an indicator of transcriptional activation. Washes in 0.1M PB, 4x for 5 min, preceded all incubations. Sections underwent the following incubations: 1% H_2O_2 in 0.1M PB, and then blocked for 60 min in 2% normal goat serum and 0.04% Triton-X in 0.1M PB (blocking solution); then rabbit anti-ERα primary antibody (1: 8,000; EMD Millipore, Billerica, MA, USA) or rabbit anti-AR (1: 400; EMD Millipore, Billerica, MA, USA) in blocking solution, overnight at room temperature. The following day, sections were incubated in anti-rabbit biotinylated secondary antibody (1:500 in blocking solution; Vector Labs, Burlingame, CA, USA) before avidin-biotin conjugate (1:000 in 0.1M PB; Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). Sections were then incubated with biotinylated tyramine (1:1000 in 0.1M PB; Perkin Elmer, Waltham, MA) for 10 min, and visualized with Alexa 488-tagged streptavidin (1:400 in 0.1M PB; Life Technologies, Grand Island, NY). After washing thoroughly with 0.1M PB, sections were then incubated with mouse anti-Fos primary antibody (1:600; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking solution, overnight at room temperature. The following day, sections were incubated for 60 minutes with Alexa 555 goat-anti mouse secondary (1:400 in 0.1M PB; Life Technologies, Grand Island, NY). Sections were then mounted and coverslipped. For negative controls, sections underwent the same immunostaining procedure in parallel, with primary antibodies excluded. Immunofluorescence was detected on a Zeiss Axio Scope.A1 microscope equipped with fluorescence channels. To determine the number of cells containing ERα or AR, Fos, and

double-labeled cells, the mPOA was identified using the anterior commissure and optic chiasm as landmarks at 20x magnification. All immunolabeled cells were counted bilaterally in a 300 × 400 μm area in the middle of the POA in six sections across the mPOA from rostral to caudal (Fig. 3.1). Counts were performed manually using ImageJ. Cell counts were averaged across both hemispheres. We use the terminology anterior (2 most rostral sections), central (2 middle sections) and posterior (2 most caudal sections) mPOA.

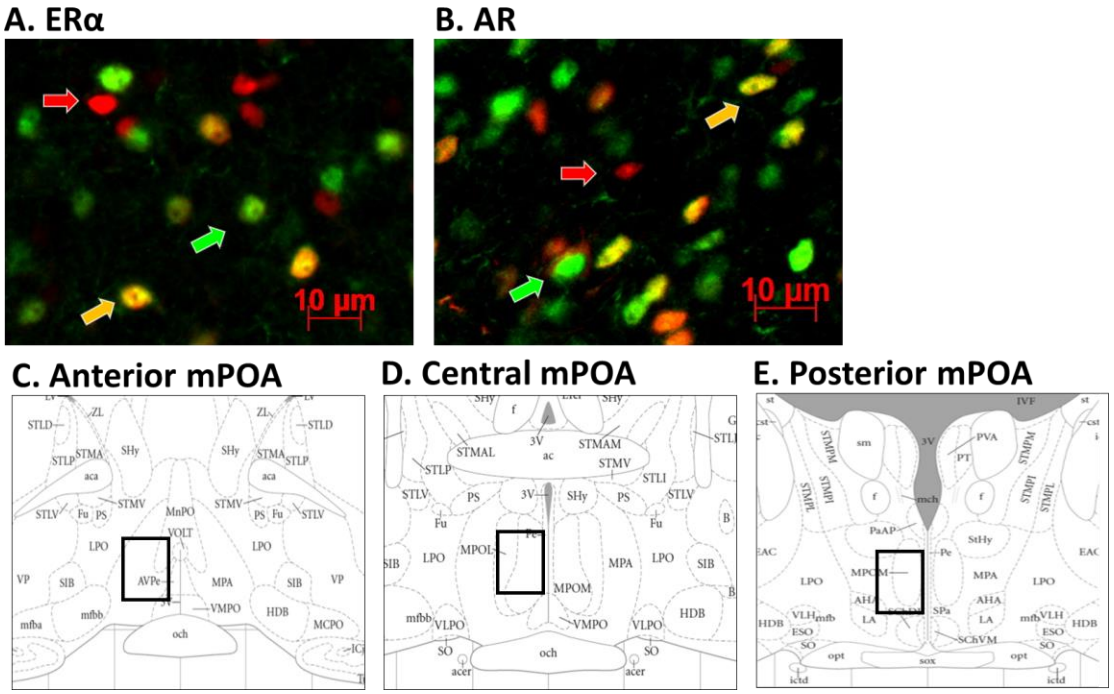

Figure 3.1 Representative micrograph of double-labeling of ERα- and Fos- in the mPOA ERα-immunoreactive cells are labeled with a green fluorophore as indicated with a green arrow. Fos–immunoreactive cells are labeled with a red fluorophore as indicated with a red arrow. Colocalized ERα-and Fos- immunoreactive cells appear yellow, and an example shown with the orange arrow. B) Representative micrograph of double-labeling of AR-(green) and Fos (red), similarly labeled as in A. Counting of single- and double-labeled cells was done in 30 x 400 um^2 sections of the mPOA at anterior (C, Bregma 0.12mm), central (D, Bregma -0.36mm), and posterior (E, Bregma -0.96) levels. Coordinates are shown with respect to Bregma from Paxinos 2007. Scale bar = 10 um..

3.3.4 Serum Estradiol

At euthanasia, a terminal blood sample was collected and placed on ice until itwas centrifuged at 4°C 1500 x g for 10 minutes. Serum was collected and stored at −80°C until analysis. All hormone assay protocols were identical to those published previously (Yin et al., 2015). Estradiol concentrations were analyzed in duplicate samples (200 μl) in a single assay, using an estradiol RIA kit (Cat. No. DSL-4800, Beckman Coulter, Webster, TX); assay sensitivity was 6 pg/mL and intra-assay CV was 4.3%. We were unable to measure serum testosterone due to a shortfall in serum volumes.

3.3.5 Statistical analysis

The mPOA is highly heterogeneous from rostral to caudal, with different levels playing differing roles in the regulation of appetitive or consummatory sexual behaviors (Balthazart and Ball 2007). Therefore, analysis was performed independently for the anterior, central, and posterior subsections of the mPOA, each represented by 2 sections per rat. First, a one-way ANOVA was used to determine if the number of ERα and AR cells differed across these mPOA subsections; Tukey's post hoc tests were used for follow up when appropriate. For subsequent analyses, two-way ANOVA was used to determine main effects of age and sexual activity, and their interactions, followed by post hoc tests when appropriate. This method was used to analyze differences in the number of cells immunopositive for ERα, AR, Fos, and the percent of Fos -positive cells that express AR or ERα. ANOVAs were performed with R (version 3.2.2) and significance

was set at $p < 0.05$. Correlations between estradiol and behaviors were done using Sigma Stat, independently on anterior, central and posterior mPOA subsections, and separately for the MAT and AG males.

3.4 RESULTS

Representative micrographs are presented in Figure 3.1, along with a map of the anterior, central, and posterior mPOA that were analyzed. First, we used one-way ANOVA to determine whether ERα, AR, or Fos cell numbers varied from anterior to posterior. For ERα, there was a significant difference in the number of ERα containing cells across the 3 subregions of the mPOA ($F_{(2,96)} = 90.32$, $p < 0.001$; Figure 2). Tukey's post hoc tests demonstrated the central mPOA had significantly more ERα-positive cells than both the posterior mPOA and the anterior mPOA. For AR, one-way ANOVA revealed there was a significant difference in the number of cells across subregions of the mPOA ($F_{(2,90)} = 188.6$, $p < 0.001$; Figure 2), with the posterior mPOA having significantly more AR-positive cells than the central mPOA, which in turn had significantly more AR-positive cells than the anterior mPOA. For Fos there was a significant difference across subregions ($F_{(2,90)} = 90.32$, $p < 0.001$; Figure 2). Tukey's post hoc tests demonstrated the central and posterior mPOA both had significantly more Fos-positive cells than the rostral mPOA.

3.4.1 Anterior mPOA

ERα: In the anterior mPOA, two-way ANOVA revealed no main effects of sexual activity ($F_{(1, 30)} = 2.70$, $p = 0.111$) or age ($F_{(1, 30)} = 2.91$, $p = 0.098$), or an age by sex interaction ($F_{(1,30)} = 3.96$, $p = 0.056$) on the number of ERα-positive cells (Fig. 3.2A).

AR: Similar to ERα, there were no main effects of either sexual activity ($F_{(1, 26)}$ = 4.12, p = 0.053) or age ($F_{(1, 26)}$ = 0.64, p = 0.429), nor was there an age by sex interaction ($F_{(1, 26)}$ = 2.29, p = 0.142; Fig. 3.2D).

Fos: There was a significant main effect of sexual activity ($F_{(1, 30)}$ = 14.31, p < 0.001) on the number of Fos-positive cells in the anterior mPOA (Fig. 3.2G), such that subjects that had sex prior to sacrifice had significantly more Fos-positive cells than their no-sex counterparts. However, there was no effect of age ($F_{(1, 30)}$ = 0.01, p = 0.927) nor was there an age by sex interaction ($F_{(1, 30)}$ = 3.42, p = 0.074).

%ERα-positive cells that co-express Fos: There was a significant main effect of sexual activity ($F_{(1, 26)}$ = 23.19, p < 0.001) but no effect of age ($F_{(1, 26)}$ = 0.08, p = 0.777) on the percent of Fos-positive cells that contained ERα, nor was there an age by sex interaction ($F_{(1, 26)}$ = 0.08, p = 0.783; Fig. 3.3A).

%AR-positive cells that co-express Fos: There was a significant main effect of sexual activity ($F_{(1, 26)}$ = 6.41, p < 0.018). There was not a main effect of age ($F_{(1, 26)}$ = 0.01, p = 0.921) on the percent of Fos-positive cells that contained ERα, nor was there an age by sex interaction ($F_{(1, 26)}$ = 0.24, p = 0.629; Fig. 3.3D).

%Fos-positive cells that co-express ERα: There was a not a main effect of either sexual activity ($F_{(1, 30)}$ = 0.72, p = 0.402) or age ($F_{(1, 30)}$ = 0.03, p = 0.853) on the percent of Fos-positive cells that contained ERα, nor was there an age by sex interaction ($F_{(1, 30)}$ = 0.20, p = 0.656; Fig. 3.4A).

%Fos-positive cells that co-express AR: Similar results were found for AR as for ERα. There was a not a main effect of either sexual activity ($F_{(1, 26)} = 1.24$, $p = 0.274$) or age ($F_{(1, 26)} = 0.88$, $p = 0.356$) on the percent of Fos-positive cells in the anterior mPOA that contained AR, nor was there an age by sex interaction ($F_{(1, 26)} = 0.76$, $p = 0.389$; Fig 3.4D).

3.4.2 Central mPOA

ERa: In the central mPOA there was a not a main effect of either sexual activity (F (1, 29) = 0.01, p = 0.943) or age (F (1, 29) = 0.77, p = 0.387) on the number of ERa-positive cells in the central mPOA, nor was there an age by sex interaction (F (1,29) = 2.70, p = 0.111; Fig. 3.2B).

AR: There was a not a main effect of either sexual activity (F (1, 27) = 0.25, p = 0.622) or age (F (1, 27) = 1.08, p = 0.308) on the number of AR-positive cells, nor was there an age by sex interaction (F (1, 27) = 0.07, p = 0.799; Fig. 3.2E).

Fos: There was a significant main effect of sexual activity (F (1, 29) = 89.99, p < 0.001) on the number of Fos-positive cells. Male rats that had sex prior to euthanasia had significantly more Fos-positive cells than the no-sex males (Fig. 3.2H). There was not a main effect of age (F (1, 29) = 0.60, p = 0.465) nor was there an age by sex interaction (F (1, 29) = 0.21, p = 0.650).

%ERa-positive cells that co-express Fos: There was a significant age by sex interaction (F (1, 29) = 5.28, p < 0.05) on the percent of Fos-positive cells in the central mPOA that co-expressed ERa (Fig. 3.3B). Specifically, post hoc analysis revealed that

AG-S animals had significantly more ERa-Fos cells than MAT-S and AG-S animals, and MAT-S animals had more ERa-Fos than MAT-NS.

%AR-positive cells that co-express Fos: There was a significant main effect of sexual activity ($F(1, 27) = 6.41$, $p < 0.05$; Fig. 3.3E) on AR-Fos cells, such that animals that had sex had significantly more cells than those that did not copulate. However, there was not a main effect of age ($F(1, 27) = 0.010$, $p = 0.921$) nor an age by sex interaction ($F(1, 27) = 0.24$, $p = 0.629$; Fig. 3.3E)

%Fos-positive cells that co-express ERa: There was a significant age by sex interaction ($F(1, 29) = 11.63$, $p < 0.01$) on the percent of Fos-positive cells in the central mPOA that co-expressed ERa (Fig. 3.4B). Specifically, post hoc analysis revealed that AG-S animals had significantly more Fos-ERa cells than all other groups.

%Fos-positive cells that co-express AR: There was a significant main effect of sexual activity ($F(1, 27) = 8.48$, $p < 0.01$; Fig. 3.4E) on Fos-AR cells, such that animals that had sex had significantly more cells than those that did not copulate prior. However, there was not a main effect of age ($F(1, 27) = 0.001$, $p = 0.991$) nor an age by sex interaction ($F(1, 27) = 0.05$, $p = 0.819$) on this endpoint..

3.4.3 Posterior mPOA

ERα: There were no significant main effects of either sexual activity ($F_{(1, 28)} = 3.98$, $p = 0.056$) or age ($F_{(1, 28)} = 1.14$, $p = 0.295$) on the number of ERα-positive cells, nor was there an age by sex interaction ($F_{(1,28)} = 0.37$, $p = 0.548$; Fig. 3.2C).

AR: There was a not a main effect of either sexual activity ($F_{(1, 28)} = 0.32$, $p = 0.578$) or age ($F_{(1, 28)} = 0.68$, $p = 0.415$) on the number of AR-positive cells, nor was there an age by sex interaction ($F_{(1, 28)} = 0.16$, $p = 0.692$; Fig. 3.2F).

Fos: There was a significant main effect of sexual activity ($F_{(1, 28)} = 36.71$, $p < 0.001$) on the number of Fos-positive cells in the posterior mPOA (Fig. 3.2I). Male rats that had sex prior to euthanasia had significantly more Fos-positive cells. However, there was not a main effect of age ($F_{(1, 28)} = 0.09$, $p = 0.769$) nor was there an age by sex interaction ($F_{(1, 28)} = 1.07$, $p = 0.309$).

%ERα-positive cells that co-express Fos: There were no main effects of either sexual activity ($F_{(1, 28)} = 2.8$, $p = 0.106$) or age ($F_{(1, 28)} = 0.02$, $p = 0.895$) on the number of ERα-positive cells, nor was there an age by sex interaction ($F_{(1,28)} = 0.06$, $p = 0.806$; Fig. 3.3C).

%AR-positive cells that co-express Fos: %AR-positive cells that co-express Fos: There was a significant main effect of sexual activity ($F_{(1, 26)} = 6.41$, $p < 0.05$). There was not a main effect of age ($F_{(1, 26)} = 0.01$, $p = 0.921$) on the percent of Fos-positive cells that contained ERα, nor was there an age by sex interaction ($F_{(1, 26)} = 0.24$, $p = 0.629$; Fig. 3.3F).

%Fos-positive cells that co-express ERα: There was a main effect of sexual activity ($F_{(1, 28)} = 5.80$, $p < 0.05$), whereby animals that did not copulate prior to euthanasia had a greater %Fos-ERα cells than did the no-sex males (Fig. 3.4C).

However, there was not a main effect of age ($F_{(1, 28)} = 0.01$, $p = 0.935$) nor an age by sex interaction ($F_{(1, 28)} = 0.09$, $p = 0.767$).

%Fos-positive cells that co-express AR: There was a significant main effect of sex ($F_{(1, 28)} = 18.36$, $p < 0.01$) where animals that had sex had significantly more Fos cells that expressed AR than animals that did not copulate prior to euthanasia (Fig. 3.4F). Additionally, there was a significant main effect of age ($F_{(1, 28)} = 4.50$, $p < 0.05$), with mature animals having significantly more Fos-AR cells than aged animals. However, there was not an age by sex interaction ($F_{(1, 28)} = 0.88$, $p = 0.356$).

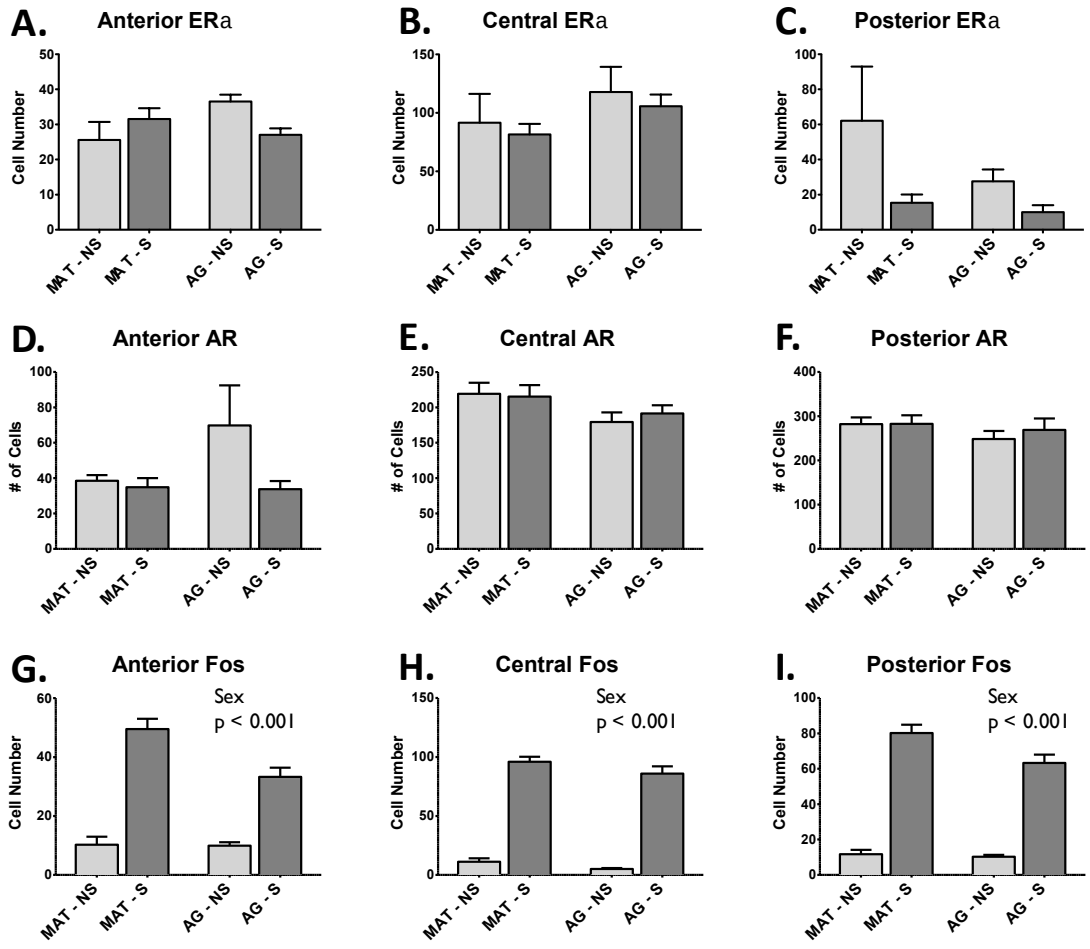

Figure 3.2 Total number of ERα, AR, and Fos in the mPOA

Total numbers of ERα (A-C), AR (D-F) and Fos (G-I) immunoreactive cell counts are shown in mature and aged male rats from the sex and no-sex groups in the three mPOA sub-regions. There were no main effects or interactions for ERα or AR in any of the sub-regions. Fos-positive cells were higher in all of the mPOA sub-regions of animals that had sex. Abbreviations here and in other figures are: MAT-S, mature-sex; MAT-NS, mature–no sex; AG-S, aged-sex; AG-NS, aged–no sex. Data here and subsequently are mean $\pm$ SEM.

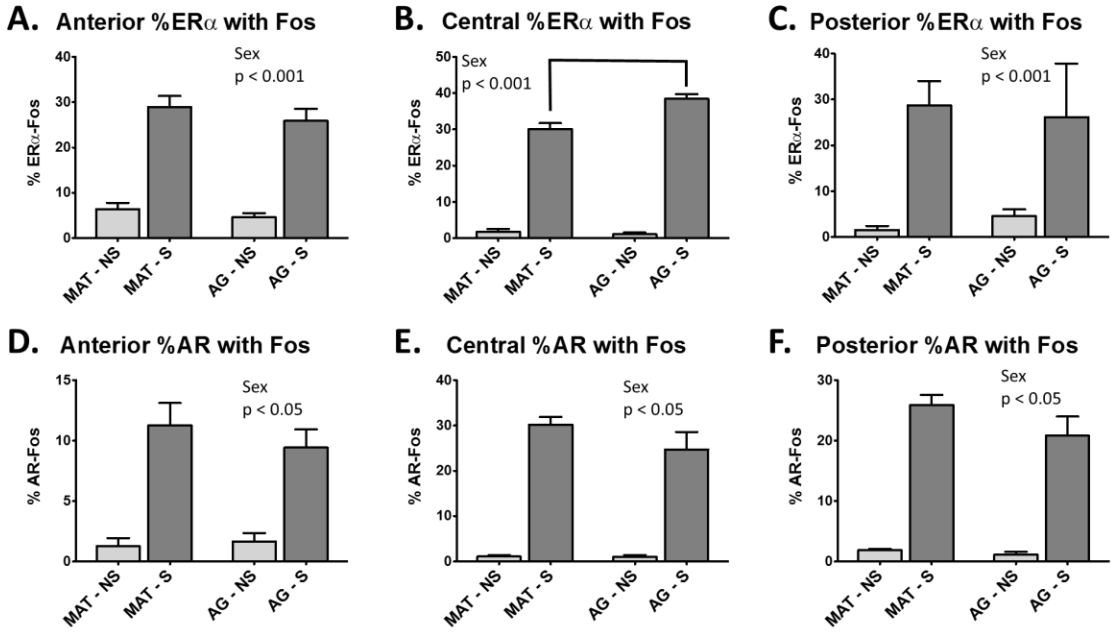

Figure 3.3 The percentage of ERα or AR cells that co-express Fos-positive are shown from anterior to posterior

For ERα-Fos double labeling (A-C), there were no effects of age, but there were main effects of sexual activity (increased compared to no sex) at all levels of the mPOA ($p < 0.001$). A similar finding was made for AR-Fos double labeling (D-F), which was significantly higher in sex than no-sex males across the mPOA.

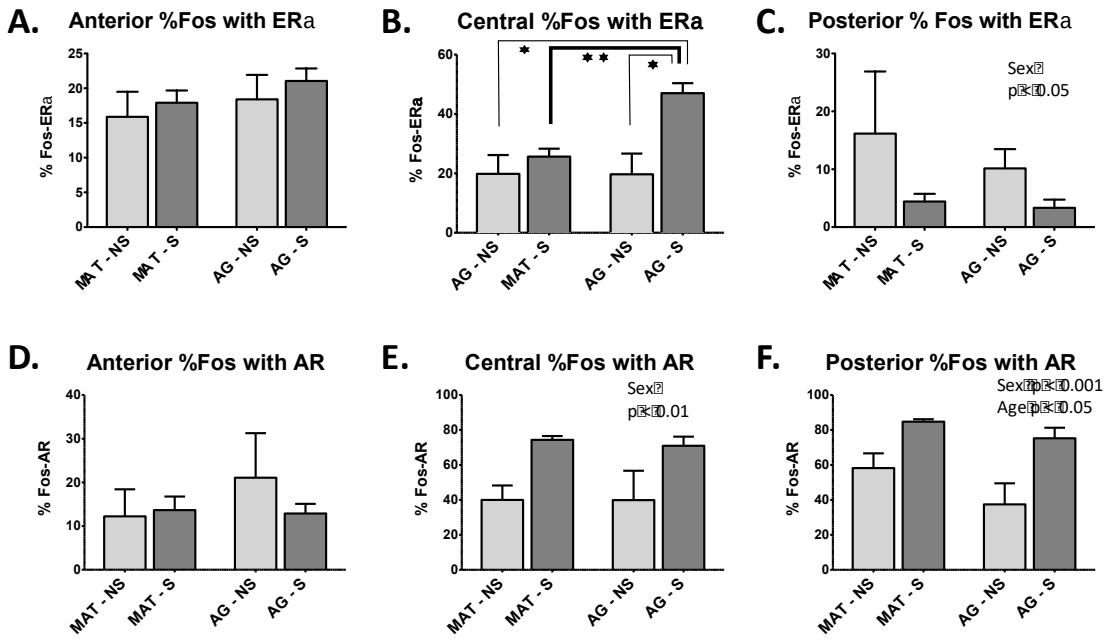

Figure 3.4 The percentage of Fos-positive cells also expressing ERα or AR are shown from anterior to posterior

For ERα-Fos double labeling, the central mPOA (B) had a significant interaction of age and sexual activity, with the AG-S group having more double-labeled cells than any other groups. In the posterior mPOA (C), there was significant main effect of sexual activity, with fewer cells in the sex than the no-sex groups. For AR-Fos double labeling, in the central mPOA (E) animals that had sex had more double-labeled cells compared to no-sex animals. In the posterior mPOA (F), main effects of both age and sexual activity were found, with higher numbers in sex vs. no-sex rats, and an age-related decrease. P-values for interactions are indicated as: *, $p < 0.05$; **, $p < 0.01$.

3.4.4 Sex Behavior

Animals that had sex prior to euthanasia had their behaviors scored for numbers of mounts, numbers of intromissions, mount latency, intromission latency and ejaculation latency. The only significant difference between MAT-S and AG-S animals was in the number of mounts (Fig. 3.5A, $p < 0.05$), for which mature animals had a greater number of mounts than aged animals. Numbers of intromissions, and behavioral latencies, were similar between the mature and aged rats

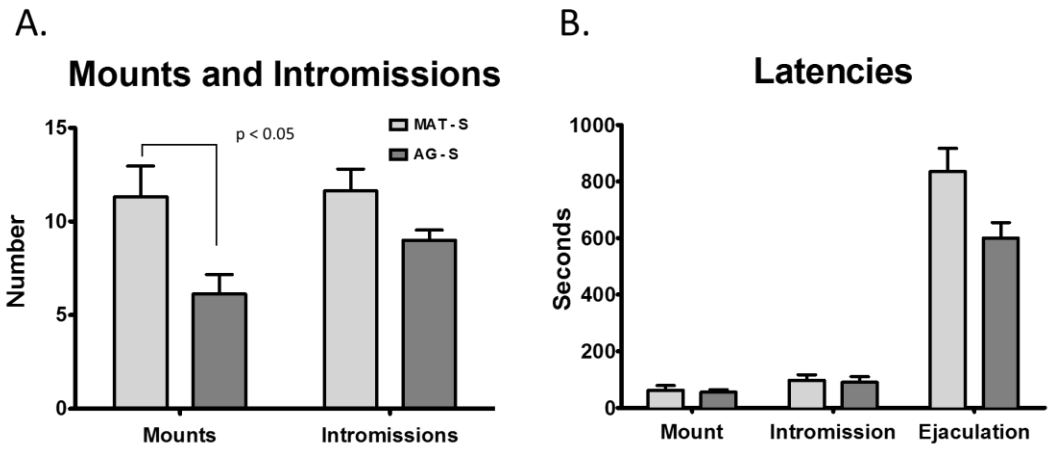

Figure 3.5 Behavioral measures are shown for MAT-S and AG-S groups

A) Numbers of mounts were significantly higher in MAT-S than AG-S males ($p < 0.05$). Numbers of Intromissions, and latencies to mount, intromit, or ejaculate (B) were unaffected.

3.4.5 Serum Estradiol Concentrations and Correlations with Behavior

A two-way ANOVA revealed there were main effects of both sexual activity ($F_{(1, 28)} = 6.116$, $p < 0.05$) and age ($F_{(1, 28)} = 4.87$, $p < 0.05$; Fig. 3.6) on serum estradiol. The AG animals had higher estradiol concentrations than MAT animals, and estradiol concentrations were higher in the sexual than the non-sex groups. Correlations were run between serum estradiol concentrations separately in the mature and aged males. Estradiol did not correlate with any behavioral measures in the mature animals. A significant positive correlation was found between estradiol and intromission latency only in aged animals ($r = 0.873$, $p < 0.01$; Fig. 3.7B). Aged animals also had a non-significant positive correlation between estradiol concentrations and mount latency ($r = .723$, $p = 0.066$; Fig 3.7A).

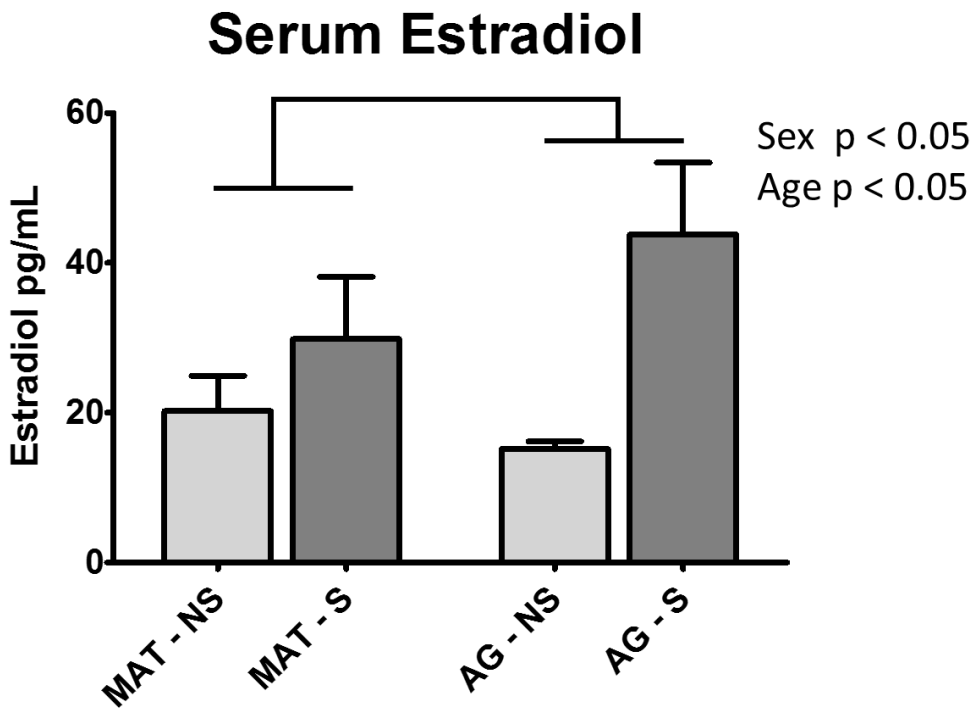

Figure 3.6 Serum estradiol concentrations

Serum estradiol concentrations are shown, measured 1 hour post-copulation or handling. There was a main effect of both sex and age ($p < 0.05$), with AG animals having higher estradiol concentrations than MAT animals, and animals that had had sex prior to sacrifice having higher estradiol concentrations than those that did not..

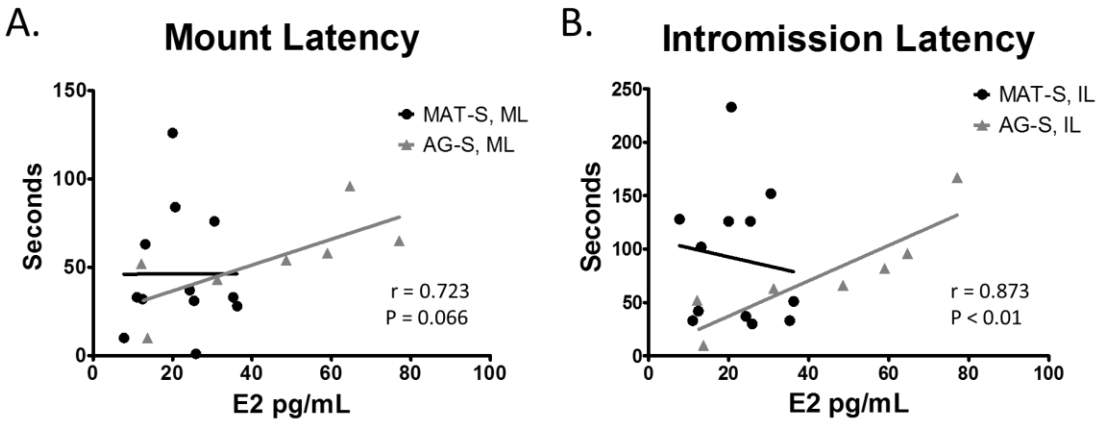

Figure 3.7 Correlations between serum estradiol and behavior

Correlations between serum estradiol and mount latency (A) and intromission latency (B) are shown separately for MAT-S and AG-S male rats. In AG-S males, mount latency had a non-significant trend for a positive correlation with estradiol ($r = 0.723$, $p = 0.066$). Intromission latency had a significant positive correlation with estradiol ($r = 0.873$, $p < 0.01$) in the AG-S males

3.5 DISCUSSION

This study examined the activation of steroid hormone receptors in the mPOA after copulation during aging males, and relationships among receptor activation, sex behavior, and peripheral serum estradiol. The sexual impairments in our aged males were relatively modest based on previous studies (Chamber and Phoenix 1984, Wu and Gore 2010, Smith et al. 1992), with the only behavioral difference a decreased mount number in aged males. However, the age of our older males at testing, 13 months, was younger than those in most other studies and we believe on the shoulder of the age-related decrement in function.

Regarding hormone receptors, we found no age-related differences in cell number for either ERα, AR or Fos in any of the sub-regions examined, but an up-regulation in Fos-immunolabeled cells in animals that copulated. When the percentage of Fos cells that co-expressed ERα or AR were examined, though, several differences with aging emerged that we believe may relate to the behavioral changes. There was also an age-related increase in the %Fos cells that co-expressed ERα in the central mPOA, and a decrease of %Fos-AR cells with age. Serum estradiol was higher both in aged animals, and animals that had sex. Estradiol did not correlate with ERα or AR cell number in either age group, or with any behavioral measures in young animals. It did correlate with mount and intromission latencies in aged animals. Taken together, our data show a particular importance for estradiol in sexual behavior in aging males

3.5.1 Sexual Experience, Steroid Hormones and Behavior

Sexual experience changes male sexual behavior, serum hormone concentrations, and hormone receptor expression. Experience alters both appetitive and consummatory

aspects of sexual behavior, increasing ultrasonic vocalizations and fos expression in the MPOA, and decreasing the number of intromissions before ejaculation and post-ejaculatory intervals (Bialy et al. 2000, Lumley and Hull 1999, Larsson 1959). Testosterone, and sometimes AR, but not estradiol or ERα, is also increased with experience (Wu and Gore 2009, Swaney et al. 2012, Hull and Dominguez 2006).

Castration eliminates sexual behavior in males, which is restored by either testosterone or estradiol plus DHT (Hull and Dominguez, 2007, McGinnis and Dreifuss 1989). DHT alone does not result in behavior significantly different from castrates, and estradiol alone facilitates some measures of sexual behavior, but usually does not restore a full copulatory sequence (Attila et al. 2009, McGinnis and Dreifuss 1989, Wu and Gore 2009, Goya et al. 1990, Roselli et al. 1993). There is evidence that there are different mechanisms controlling different components of sexual behavior (reviewed in Hull and Dominguez 2007). Estradiol is particularly important in partner preference, sexual motivation and mounting (Hamson et al. 2008, Yeh et al. 2009, Bakker et al. 2002, Roselli et al. 2003, Roselli 1999). In fact, testicular feminized (TFM) mice still prefer estrous over non-estrous females, and have normal mount latencies, indicating a functional androgen receptor may not be required for these behaviors (Hamson et al. 2008). Aromatase knockout mice also showed increased latencies to mount, intromit or ejaculate, and showed no partner preference at all (Bakker et al. 2002). Androgen receptor activation has been implicated more in ejaculation and satiety (Romano-Torres 2007, Yeh 2009). Erection also is more dependent on circulating androgens than estradiol (Hull and Dominguez 2007).

3.5.2 Steroid Hormones, Sex, and Aging

Testosterone and sexual behavior both decline during aging, but the concentration of testosterone does not predict behavior in aged animals, and testosterone administration to aged castrates does not restore behavior to that similar to young males (Wu and Gore 2009, Smith et al. 1992, Goya et al. 1990, Roselli et al. 1993, Chambers and Phoenix 1986). While in young animals, or young and old combined groups, testosterone levels cannot be used to predict performance previous research has also found facilitation of mount latency by testosterone only in aged animals (Wu and Gore 2010). We would have liked to have been able to measure serum testosterone, we predict it would not have changed drastically between our mature and aged groups due to the fact that we saw no changes in the number of intromissions.

Estradiol concentrations during aging have been less consistent than those with testosterone, either increasing, decreasing, or remaining the same (Luine et al. 2007, Herath et al. 2001, Wu and Gore 2009, Goya et al. 1990, Smith et al. 1992, Herrera-Perez et al. 2008). An age related elevation in estradiol may be due to increased aromatization in adipose tissue (Gautier et al. 2013). We also found increased estradiol in males of both ages after sexual behavior. Measuring estradiol immediately after sexual behavior is not common in males, but it has been found that they may experience a reflexive release of LH and testosterone, both upon the introduction of a female and after ejaculation (Nyby 2008, Shulman and Spritzer 2014, Kamel and Frankel 1978). Increased serum testosterone would be available for aromatization in adipose tissue, or more estradiol may be secreted directly by the testes in response to the LH surge (Winters and Troen 1986).

In these studies reflexive testosterone release did not correlate with any measure of male sexual behavior, except for the fact that it was absent in males failing to copulate, leading some to suggest its involvement in sexual satiety (Kamel and Frankel 1978, Nyby 2008). The purpose of reflexive release isn't known, as lower fixed concentrations of testosterone are sufficient for copulation, but that it is highly conserved indicates it does have some function (Bonson and Desjardins 1982, Maruniak and Bronson 1976, Nyby 2008).

3.5.3 AR and ERα Activation in Sexual Behavior and Aging

We found no age-related changes in either ERα or AR in any of the subregions in the MPOA. This is consistent with previous studies of ERα in the MPOA in males that also found no age-related changes in ERα mRNA or protein (Madeira et al. 2000, Bottner et al.2007, Wu and Gore 2010). Previous studies have found either increased (Wu and Gore 2009), decreased (Chambers et al. 1991) or no change (Wu and Gore 2010) in AR.

Previous work in young animals showed about 30% of Fos cells that were activated by mating also expressed ERα (Greco et al. 1998). Though they did not measure Fos with AR, they did examine colocalization of ERα and AR. Approximately 80-90% of ERα expressing cells also contained AR, making it likely that many ERα cells in this study also contain AR. Though they did not examine mating induced activation, Wood and Newman (2008) also found AR and ERα colocalization in hamster nuclei that both have high steroid hormone concentrations and are important in male sexual behavior, including the mPOA, BnST, and MeA. In the mPOA they found regional differences in cells expressing only ERα, where these neurons were found in increased

numbers in the rostral mPOA. Though we cannot say whether the cells we counted expressed AR, ERα or both, we did find more ERα was expressed in the central MPOA than the caudal.

3.5.4 Conclusion

We initially hypothesized that decreased activation of ERα and AR, or changes in receptor expression, in the mPOA may be contributing to sexual impairments in aged males. Our results show that not only was receptor expression largely the same across the mPOA in mature and aged animals, but activation of AR and ERα was similar. The fact that impairments of sexual behavior in aged animals were limited to numbers of mounts suggests that our animals at 13 months are on the cusp of the transition into reproductive senescence. What is most interesting is that at this point in life, serum estradiol concentrations are increasing, motivational behaviors (mounting) are decreasing, and a relationship emerges between serum E2 and latencies to intromit (and a trend to mount). These results can also be related to findings of Fos cells that co-express ERα. In the central POA, there is a substantial and significant increase in %Fos-ERα cells only in the aged males, that when considered together with the serum hormone and behavior data, suggests this subpopulation of cells as a neuroanatomical substrate for this change.

Chapter 4: Aging and Estradiol Effects on Gene Expression in the Medial Preoptic Area, Bed Nucleus of the Stria Terminalis, and Posterodorsal Medial Amygdala of Male Rats

Data presented in this chapter is under review.

4.1 ABSTRACT

Studies on the role of hormones in male reproductive aging have traditionally focused on testosterone, but estradiol also plays important roles in the control of masculine physiology and behavior. Our goal was to examine the effects of E2 on the expression of genes in brain regions selected because they are both E2-sensitive and involved in behavioral neuroendocrine functions that are impaired with aging. Mature adult (MAT, 5 mo.) and aged (AG, 18 mo.) sexually-experienced Sprague-Dawley male rats were castrated, implanted with either vehicle or estradiol (E2) subcutaneous capsules, and euthanized one month later. Bilateral punches were taken from the bed nucleus of the stria terminalis (BnST), posterodorsal medial amygdala (MePD) and the preoptic area (POA). RNA was extracted, and expression of 48 genes analyzed by qPCR using Taqman low-density arrays (TLDA). While all three regions showed changes in gene expression with E2, effects on individual genes were age- and region-specific. In the POA, 5 genes were identified, all of which increased with E2. The BnST showed primarily age-related changes with 5 genes decreasing with age, and one increasing with age. The MePD had 16 genes with a significant interaction between age and E2, and 5 with a main effect of treatment, the latter increased in E2 compared to Vehicle males. Significantly affected genes in the MePD included nuclear hormone receptors, neurotransmitters and neuropeptides and their receptors. Serum levels of hormones were also assayed. Estradiol

decreased LH and TSH. Three hormones, E2, FSH and GH had main effects of age and hormone. An interaction between age and hormone was found for BDNF, PRL and T4. These results support the idea that the male brain is highly sensitive to estradiol in an age-dependent manner, that E2 regulates expression nuclear hormone receptors, neurotransmitters and neuropeptides and their receptors of suites of genes in a region-dependent manner, and suggest that peripheral hormones are likely involved in both feed-forward and feedback effects of E2 in an age-dependent manner.

4.2 INTRODUCTION

The hypothalamus is the body's interface between the brain and the peripheral endocrine systems. Hypothalamic nuclei and their inputs and outputs from other limbic brain regions (e.g., bed nucleus of the stria terminalis (BnST), amygdala) are involved in the regulation of reproduction, mood, and memory, in part through actions of gonadal steroid hormones on their receptors in the brain (Smith et al, 1992; Hogervorst et al., 2005; Walf et al., 2009; Arimoto et al., 2013; Rosario et al.,2010). The principal estrogen, estradiol (E2), has been extensively studied for age-related changes in neurobiological actions in females, but to a much lesser extent in males (Walf et al., 2009; Harburger et al., 2009; Raber 2008; Russell et al., 2012;f Foster 2012). Furthermore, the literature on release and concentration of serum E2 in male rodents and humans with aging is inconsistent; various groups have reported increases (Fujita et al., 1990; Herath et al., 2001; Luine et al., 2007; Lakshman et al., 2010; Jasuja et al., 2013), decreases (Wu and Gore, 2009a; Khosla et al., 2001; Leifke et al., 2000; Van den Beld et al., 2000; Yeap et al., 2012), or no change (Goya et al., 1990; Gruenewald et al., 1994; Yeap et al., 2014)

with advanced age. Furthermore, research on age-related changes in estradiol's actions on male neuroendocrine systems is limited; this is an important area of study considering the profound effects that E2 plays on the aging female brain (Yin et al., 2015; Rao et al, 2013).

The preoptic area (POA), posterodorsal medial amygdala (MePD), and BnST, brain areas important in reproductive function in males, all have high concentrations of hormone receptors, including estrogen receptors (ERs) (Perez et al., 2003; Shughrue et al., 1997; Mitra et al., 2003). Changes in ERs or in estrogenic regulation of genes in these areas may underlie age-related deficits in sexual behavior and reproductive physiology in both sexes (Navarro et al., 2013; Izumo et al., 2012; Putnam et al., 2005; Yamaguchi and Yuri, 2012). When bound to the nuclear ERα or ERβ, E2 regulates genes and proteins associated with steroid hormone signaling, neurotransmission, and neuroendocrine functions. During aging, receptor density and binding affinity for the ERs, and ERα protein expression, change in a sex, region, and age-specific manner (Haji et al., 1981; Zhao et at., 2007; Thakur and Sharma 2007; Wu and Gore 2009b, Wu and Gore 2010, Roselli et al., 1993). Furthermore, E2 may also act upon membrane ERs such as GPER; a recent study showed increased density of GPER-positive cells in two hypothalamic-preoptic regions in aging compared to young adult female rhesus monkeys (Naugle et al., 2014).

How estradiol may exert its influence more broadly, beyond direct effects on ERs in hypothalamic and limbic brain regions, and the influence of aging, is not well-understood. To investigate this, we profiled expression of 48 genes in the POA, MePD

and BnST of young adult and aging male rats that were castrated and given E2 or vehicle. Our hypothesis was that the aging brain would lose responsiveness to E2, and that each region of interest would have unique E2- and age- related neuromolecular phenotypes.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Forty Sprague-Dawley male rats (Harlan, Indianapolis, IN; 3 or 12 months old at arrival) were housed individually in large plastic cages, in a climate-controlled room, on a 14:10 hr light– dark cycle (lights off at 10:00 a.m). Food and water were freely available. The older males were retired breeders. To give the younger males sexual experience, these animals were each given a 90 minute mating session with a receptive female every other day for 6 sessions. On a final 7th session, males were tested to ensure that they met criteria of two ejaculations in one hour. All of the males met these criteria and were included in the study. Castration surgery was conducted under isoflurane anesthesia when rats were 3 months or 16 months of age. One month later, at 4 or 17 months, males were implanted with 12 mm Silastic capsules (1.98 I.D. x 3.18 O.D.) containing either estradiol (5% in cholesterol), or cholesterol (100% cholesterol). One month after hormone implantation, when rats were mature (MAT, 5 months) or aged (AG, 18 months), animals were rapidly decapitated, blood samples were collected, and their brains were removed and cut into 1 mm coronal sections on a chilled brain matrix before freezing on slides and storage at -80 degrees. Bilateral punches of the POA, BnST and MePD were later taken using a 0.98 mm Palkovits punch and transferred to frozen microfuge tubes. All procedures were done in accordance with the National Institutes of

Health guidelines for the care and use of laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

4.3.2 RNA expression

RNA was extracted from frozen POA, BnST, and MePD punches using a Allprep DNA/RNA mini-kit (QIAGEN, Valencia, California), according to the manufacturer's protocols. This and other molecular work was conducted as per standard laboratory protocols (Yin et al., 2015; Walker et al., 2013). RNA (200 ng) was converted to single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems), using the manufacturer's protocol, and stored at −20°C. RNA samples were eluted with nuclease-free water and treated with 1 U of TURBO deoxyribonuclease (Applied Biosystems Inc, Foster City, California) to rid samples of genomic DNA before ethanol precipitation. Resuspended samples were diluted to a concentration of 50 ng/µL. The purity, integrity and concentration of all samples were tested by running them on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California) and a nanodrop. All samples had RINs of 8 or higher.

Samples (n = 8-10 per group) from the BnST, MePD, and POA were run on customized rat Taqman low-density array (TLDA) Microfluidic 48-gene real-time PCR cards (Applied Biosystems) as described (Walker et al., 2013) with specific gene assays chosen based on *a priori* hypotheses and publications on their importance in neuroendocrine function and reproductive aging (46 genes of interest and two normalizing genes; Figure 4.1).

Ar	Androgen receptor
Avpr1a	Arginine vasopressin receptor 1a
Avp	Arginine vasopressin
Bdnf	Brain-derived neurotrophic factor
Crh	Corticotropin releasing hormone
Cyp19a1	Cytochrome p450
Dbh	Dopamine beta-hydroxylase
Drd1a	Dopamine receptor d1
Drd2	Dopamine receptor d2
Drd3	Dopamine receptor d3
Drd4	Dopamine receptor d4
Esr1	Estrogen receptor 1
Esr2	Estrogen receptor 2
Glo1	Glyoxalase 1
Gper	G protein-coupled estrogen receptor 1 (GPR30)
Grin2a	Glutamate receptor, ionotropic, N-methyl-D-aspartate 2a (NR2a)
Grin2b	Glutamate receptor, ionotropic, N-methyl-D-aspartate 2b (NR2b)
Grm1	Glutamate receptor, metabotropic 1
Grm5	Glutamate receptor, metabotropic 5
Gsr	Glutathione reductase
Htr1a	5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled
Kiss1	Kisspeptin
Kiss1r	Kisspeptin receptor
Lepr	Leptin receptor

Mc3r	Melanocortin 3 receptor
Mc4r	Melanocortin 4 receptor
Mc5r	Melanocortin 5 receptor
Ncoa2	Nuclear receptor coactivator 2
Nos1	Nitric oxide synthase 1 (neuronal)
Npvf	Neuropeptide VF precursor
Npy	Neuropeptide Y
Nr3c1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2
Oxt	Oxytocin
OxtR	Oxytocin receptor
Pdyn	Prodynorphin
Pgr	Progesterone receptor
Pomc	Proopiomelanocortin
Slc6a3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
Slc6a4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
Tac2	Tachykinin 2
Tac3	Tachykinin 3
Thra	Thyroid hormone receptor, alpha
Thrb	Thyroid hormone receptor, beta
Th	Tyrosine hydroxylase
Trh	Thyrotropin releasing hormone

Figure 4.1 Supplemental Table 1

Genes on the 48 Gene qPCR TLD Card. Drd4 failed to amplify, so it is excluded here and in other. Two additional genes, 18s and Gapdh were also measured and Gapdh used for normalization.

Real-time PCR was performed using Taqman universal mastermix (Applied Biosystems) on an ABI ViiA7 machine with the following run parameters: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Relative expression of each gene was determined using the comparative cycle threshold method (Pfaffl, 2001; Schmittgen & Livak, 2008). Each sample was normalized to *Gapdh* expression and then calibrated to the median δ-cycle threshold of the lowest-expressing group within each gene.

4.3.3 Serum Hormones

After collection blood was centrifuged at 2300 x g for 5 min. Serum was collected and stored at −80°C until analysis. All hormone assay protocols were identical to those published previous (Yin et al., 2015). Estradiol was analyzed in duplicate samples (200 ul) in a single assay, using an estradiol RIA kit (Cat. No. DSL-4800, Beckman Coulter, Webster, TX); assay sensitivity was 6 pg/mL and intra-assay CV was 1.2%. Concentrations of peripheral protein/peptide hormones were measured in duplicate 10 ul serum samples in a single Milliplex rat pituitary magnetic bead assay (RPTMAG-86K, Millipore, Billerica, MA). Intra-assay CV for each hormone was: luteinizing hormone (LH) 0.85%, follicle-stimulating hormone (FSH) 5.3%, thyroid stimulating hormone (TSH) 4.3%, adrenocorticotropic hormone (ACTH) 2.2%, growth hormone (GH) 3.2%, prolactin (PRL) 1.5%, and brain-derived neurotrophic factor (BDNF) 2.1%. Rat steroid Magnetic Bead assay (STTHMAG-21K; Millipore) was used to measure serum progesterone, triiodothyronine (T3) and thyroxine (T4), with intra-assay CV of progesterone 8.1%, T3

11.4%, and T4 7.6%. The Magnetic Bead assays were conducted in the laboratory of Dr. Andrew Wolfe, Johns Hopkins University School of Medicine, and the E2 RIA in the Gore Lab at the University of Texas at Austin.

4.3.4 Data Analysis

Gene expression data were normalized to *Gapdh* within each animal, expressed relative to the MAT vehicle group. A Grubb's test for outliers was conducted, and up to two outliers per group were removed if indicated. A two-way analysis of variance was performed to test for main effects of age, hormones, and interactions, followed by a Bonferonni post-hoc analysis, when indicated. When data failed tests for normality and homogeneity, they were square root transformed and analyzed with an ANOVA. If transformation did not resolve normality issues, un-transformed data were analyzed with a non-parametric Kruskal-Wallis test. In this case, main effects were tested for with t-tests, and an interaction was identified by testing for the effects of one treatment while holding the other constant, and vice versa. Data analyses were performed with Sigma Stat 7. Gene expression heatmaps were created to visualize relationships in gene expression across experimental groups. In order to cluster genes based on expression, a Pearson pairwise correlation matrix was generated for all genes across all treatment groups within each brain region (mPOA, BnST, MePD). A distance matrix was then created by subtracting one from the absolute value of each pairwise correlation, and complete linkage hierarchical clustering was performed based on this distance matrix. A hierarchical dendrogram was then generated for each heatmap based on the clustering results. To visualize gene expression differences between groups on the heatmap, gene

expression values within a group were normalized as z-scores. Treatment groups were then hierarchically clustered based on Euclidian distance between these values. Heatmap construction (heatmap.2 in *gplots*) and clustering were performed in R (3.1.2). An effect was considered significant at $p < 0.05$..

4.4 RESULTS

4.4.1 Gene Expression in the POA

Of the 46 genes of interest, five had a significant effect of E2 treatment: *Avpr1a*, *Mc3r, Oxt*, *Oxtr*, *Mc3r,* and *Bdnf* (Figure 4.2). In all five cases, E2 treated male rats showed higher gene expression than Vehicle groups. There were no significant main effects of age, or any significant hormone by age interactions, for any genes in the POA. Detailed results for genes not significantly affected in the POA are provided Figure 4.3.

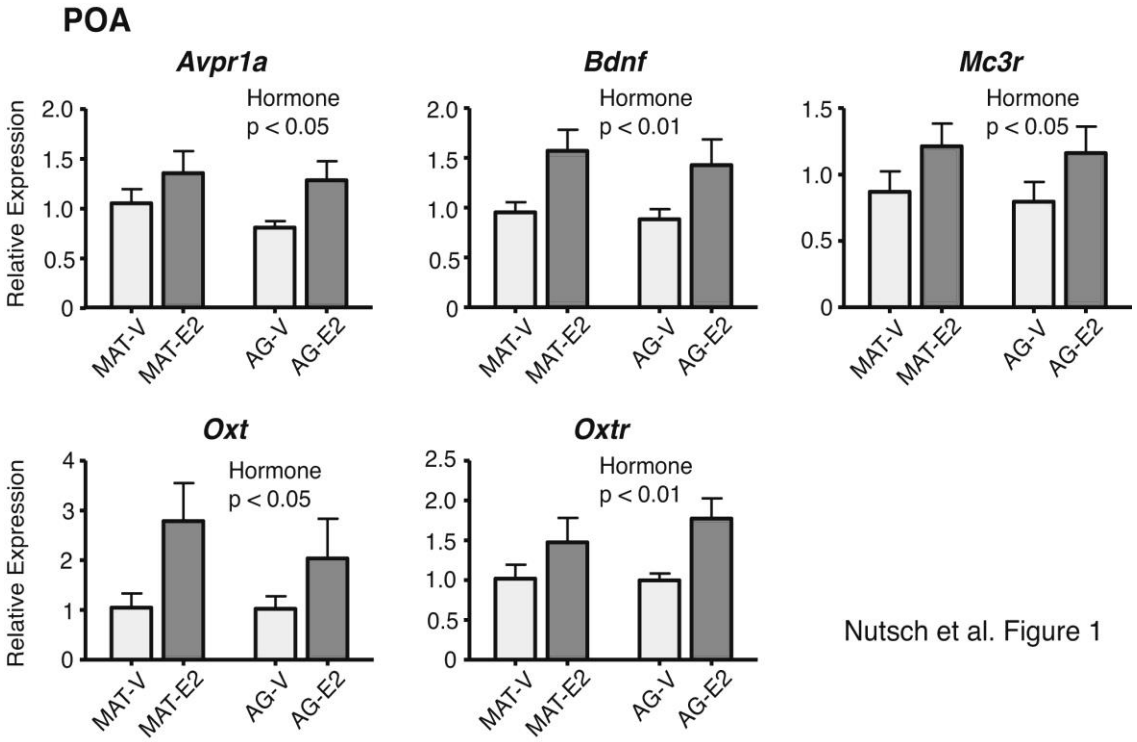

Figure 4.2 Effects of hormone administration in the POA.

Genes significantly affected by hormone treatment are shown for the POA. All five of these genes were higher in E2 than Vehicle male rats. There were no interactions or age related differences. P-values for significant main effects of hormone treatment are indicated. Abbreviations here and in other figures are: MAT-V, mature-vehicle; MAT-E2, mature-estradiol; AG-V, aged-vehicle; AG-E2, aged-estradiol.

POA results for non-significant genes

	MAT-Vehicle		MAT-E2		AG-Vehicle		AG-E2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ar	0.94	0.12	1.12	0.11	1.00	0.12	1.19	0.16
Avp	2.72	1.03	7.40	2.36	1.74	0.62	11.78	6.26
Crh	1.17	0.24	1.83	0.76	1.18	0.18	1.18	0.44
Cyp19a1	1.16	0.23	1.87	0.42	1.18	0.25	1.40	0.28
Dbh	4.88	2.73	1.64	0.54	N/D		N/D	
Drd1a	8.51	6.68	1.42	0.30	2.12	1.38	1.05	0.20
Drd2	1.67	0.57	1.30	0.44	1.06	0.25	0.68	0.11
Drd3	1.46	0.38	1.25	0.34	1.84	0.87	0.87	0.21
Esr1	1.08	0.23	1.45	0.45	1.03	0.16	0.93	0.24
Esr2	0.93	0.12	1.07	0.22	0.99	0.11	0.99	0.16
Glo1	0.98	0.07	1.19	0.18	0.93	0.07	1.05	0.08
Gper	0.95	0.13	1.40	0.34	0.95	0.08	1.04	0.08
Grin2a	1.50	0.35	1.54	0.36	1.10	0.18	0.97	0.09
Grin2b	1.45	0.31	1.62	0.43	1.01	0.12	1.14	0.10
Grm1	0.96	0.09	0.95	0.22	1.04	0.13	0.95	0.17
Grm5	1.14	0.19	1.12	0.22	0.86	0.09	0.98	0.08
Gsr	0.97	0.10	2.56	1.37	0.85	0.05	1.01	0.11
Htr1a	1.01	0.09	1.38	0.26	1.22	0.23	1.44	0.15
Kiss1	1.54	0.61	22.79	7.00	1.33	0.47	12.27	4.89
Kiss1r	0.88	0.13	0.89	0.04	0.90	0.13	0.83	0.10
Lepr	1.12	0.16	0.95	0.10	1.20	0.14	1.37	0.20
Mc4r	1.07	0.18	3.54	2.03	0.98	0.11	0.60	0.08
Mc5r	2.60	1.59	14.58	13.64	0.69	0.11	1.03	0.24
Ncoa2	1.03	0.10	1.35	0.33	0.97	0.12	0.92	0.07
Nos1	1.11	0.12	1.75	0.47	1.19	0.22	1.16	0.27
Npvf	2.29	0.89	4.30	1.05	1.78	0.34	19.19	12.68
Npy	3.23	2.21	1.87	0.96	1.04	0.42	1.51	0.74
Nr3c1	0.95	0.09	1.25	0.29	0.94	0.03	1.00	0.08
Ntrk2	1.08	0.15	2.72	1.34	0.84	0.07	1.01	0.11
Pdyn	1.46	0.43	1.56	0.32	1.15	0.09	1.47	0.33
Pgr	1.03	0.13	3.16	1.02	0.95	0.05	1.51	0.27
Pomc	1.25	0.29	51.13	30.87	0.54	0.09	38.47	24.20
Slc6a3	1.42	0.39	2.32	0.42	1.24	0.27	1.91	0.60
Slc6a4	1.64	0.58	1.68	0.60	1.62	0.73	0.87	0.13
Tac2	1.24	0.26	1.77	0.59	1.35	0.14	1.09	0.26
Tac3	1.28	0.23	1.24	0.32	1.34	0.47	1.03	0.42
Thra	1.01	0.08	1.20	0.29	0.98	0.05	0.97	0.08
Thrb	1.04	0.10	1.13	0.14	0.95	0.08	1.08	0.13
Th	1.21	0.35	1.72	0.30	1.01	0.20	1.03	0.22

Figure 4.3 Supplemental Table 2

Expression of non-significant genes in the POA. Relative expression (mean + SEM) is shown for those genes not significantly affected by E2, age, or their interaction. N/D = Not detectable (did not amplify).

4.4.2 Gene expression in the BnST

In the BnST a significant main effect of age was found for *Cyp19a1, Esr2, Nos1* and *Thra,* such that mature rats had higher gene expression than the aged rats (Figure 4.4). *Kiss1r* was also affected by age, but was higher in the aged than mature males. *Oxtr* had significant main effects both of age and hormone ($p < 0.05$ and $p < 0.01$ respectively), with expression lower in the aged than the mature males, and higher in E2 than Vehicle groups. *Grm5* had a significant interaction of E2 and age ($p < 0.05$). Detailed results for genes not significantly affected in the BnST are provided in Figure 4.5.

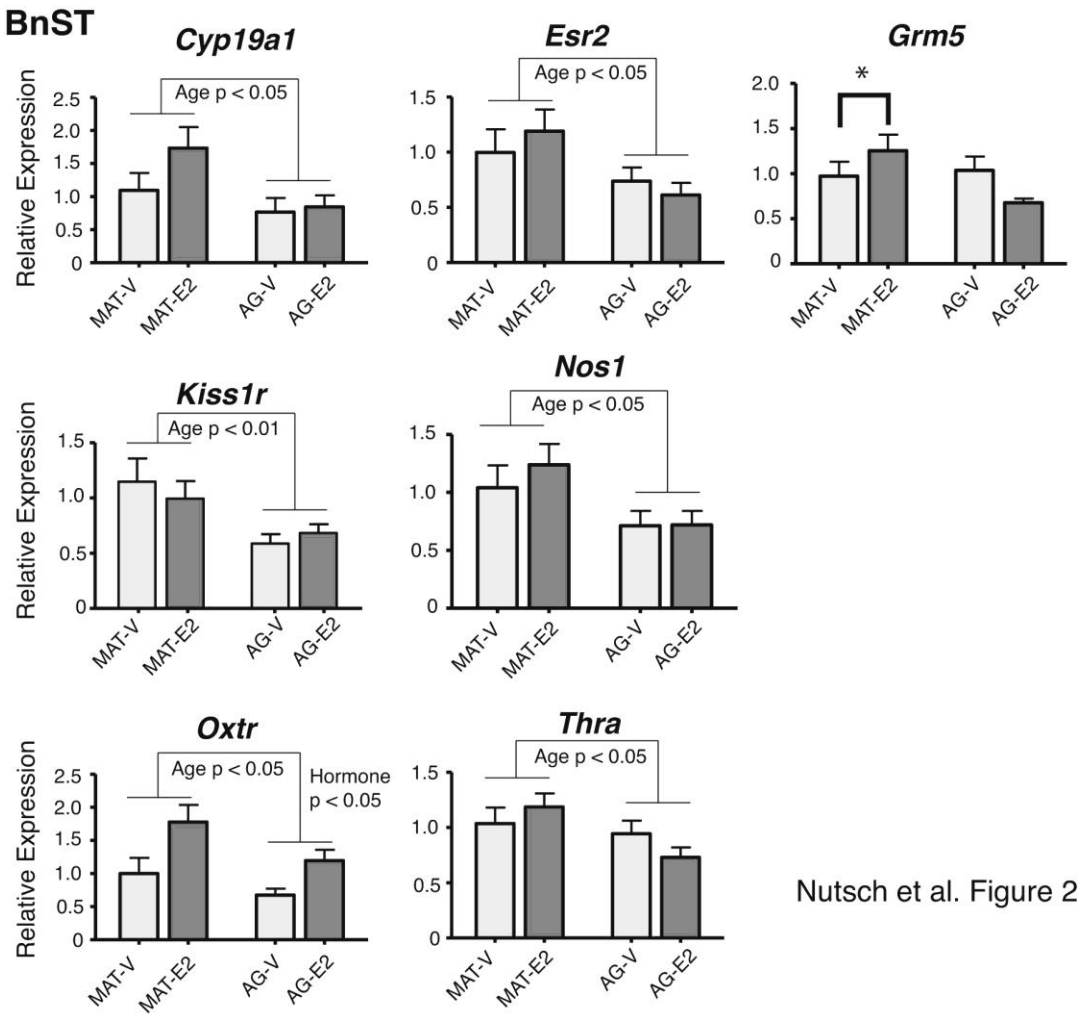

Figure 4.4 Effects of age in the BnST.

Genes significantly affected by age, hormone, and their interactions are shown for the BnST. Significant age effects were found for *Cyp19a1*, *Esr2*, *Kiss1r*, *Nos1* and *Thra*, all lower in aged than mature male rats. *Oxtr* was decreased in aged compared to mature animals, and increased in E2 compared to Vehicle animals ($p < 0.05$ for both). *Grm5* had a significant interaction of age and hormone, attributable to the mature-E2 rats having higher expression than the aged-E2 rats (indicated by bold bracket and *). P-values for significant main effects are indicated.

BnST results for non-significant genes

	MAT-Vehicle		MAT-E2		AG-Vehicle		AG-E2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ar	1.19	0.27	1.63	0.24	0.98	0.17	1.04	0.17
Avpr1a	0.84	0.13	0.79	0.10	0.84	0.10	0.67	0.08
Avp	1.14	0.34	0.74	0.13	1.07	0.32	0.85	0.25
Bdnf	0.86	0.11	0.98	0.24	1.61	0.94	0.74	0.15
Crh	1.43	0.64	1.38	0.24	1.26	0.46	0.94	0.46
Dbh	N/D		N/D		N/D		N/D	
Drd1a	1.29	0.50	1.04	0.15	3.11	1.25	0.54	0.17
Drd2	1.02	0.26	1.57	0.24	3.34	1.66	0.80	0.30
Drd3	0.98	0.13	0.89	0.10	0.97	0.17	0.56	0.03
Esr1	1.07	0.25	1.15	0.20	0.84	0.19	0.75	0.24
Glo1	0.99	0.09	0.95	0.08	1.02	0.09	0.77	0.06
Gper	0.88	0.06	0.87	0.09	1.46	0.59	0.59	0.04
Grin2a	0.89	0.10	0.92	0.12	1.04	0.23	0.67	0.13
Grin2b	1.48	0.44	1.11	0.18	0.96	0.10	0.72	0.06
Grm1	0.92	0.11	0.91	0.11	0.95	0.19	0.68	0.10
Gsr	0.99	0.08	0.94	0.10	1.26	0.37	0.69	0.02
Htr1a	1.10	0.33	0.93	0.19	0.73	0.20	0.55	0.08
Kiss1	1.10	0.17	5.10	1.48	2.27	0.53	4.84	2.63
Lepr	0.95	0.22	0.63	0.10	1.36	0.30	1.24	0.41
Mc3r	1.18	0.36	1.52	0.36	4.60	3.54	1.47	0.76
Mc4r	0.87	0.18	0.94	0.12	2.12	1.43	0.60	0.16
Mc5r	0.85	0.10	1.68	0.40	17.53	16.48	0.88	0.11
Ncoa2	1.11	0.16	1.34	0.18	1.11	0.10	0.93	0.06
Npvf	2.34	0.65	2.37	0.26	1.80	0.55	2.26	0.13
Npy	1.02	0.20	1.16	0.20	2.72	1.74	0.70	0.14
Nr3c1	1.10	0.13	1.36	0.22	1.08	0.09	0.79	0.07
Ntrk2	0.89	0.15	1.09	0.16	1.51	0.70	0.65	0.06
Oxt	0.86	0.24	0.94	0.16	0.40	0.06	0.70	0.32
Pdyn	0.85	0.16	1.16	0.19	1.65	0.67	0.78	0.23
Pgr	1.10	0.17	1.17	0.12	1.33	0.40	1.04	0.25
Pomc	1.05	0.18	7.89	7.01	0.56	0.10	1.24	0.89
Slc6a3	1.91	0.49	1.77	0.26	1.49	0.26	3.50	1.40
Slc6a4	1.05	0.20	1.02	0.13	N/D		1.14	0.17
Tac2	1.09	0.36	1.29	0.25	1.49	0.63	0.92	0.38
Tac3	1.03	0.23	1.23	0.20	0.94	0.30	0.61	0.12
Thrb	1.07	0.14	1.16	0.11	1.02	0.12	0.77	0.10

Figure 4.5 Supplemental Table 3

Expression of non-significant genes in the BnST. Relative expression (mean + SEM) is shown for those genes not significantly affected by E2, age, or their interaction. N/D = Not detectable (did not amplify).

4.4.3 Gene Expression in the MePD

The MePD had the largest number of significant changes. Significant main effects of age were found for expression of *Bdnf, Glo1, Gsr, Thrb* and Grin2b, all of which were higher in aged than mature rats (Figure 4.6). *Grin2b* was also affected by hormone treatment, higher in E2 than Vehicle males ($p < 0.05$). Seventeen genes had a significant age by hormone interactions: *Ar, Avp, Cyp19a1, Esr1, Esr2, Grm1, Kiss1r, Mc3r, Nos1, Npy, Nr3c1, Ntrk2, Oxt, Oxtr, Tac3, Th,* and *Trh,* ($p<.05$, Figure 4.7). Of these, *Avp* also had a significant main effect of hormone (higher in E2 than vehicle males). Although *Gper* showed a significant interaction using a two-way analysis of variance, it failed to show any significant post-hoc changes between groups. For the other genes with significant interactions, it is notable that many that were up-regulated by E2 in the mature males were down-regulated by E2 in the aged males. A summary of result for the POA, BnST and MePD is shown in Figure 4.8. Interestingly, each region had unique expression patterns, with POA genes affected only by hormone; the BnST primarily by age; and the MePD by interactions of age and hormone. The only gene significantly affected in all 3 regions was Oxtr, although the directionality of effects differed between the regions. Detailed results for genes not significantly affected in the MePD are provided (Figure 4.9).

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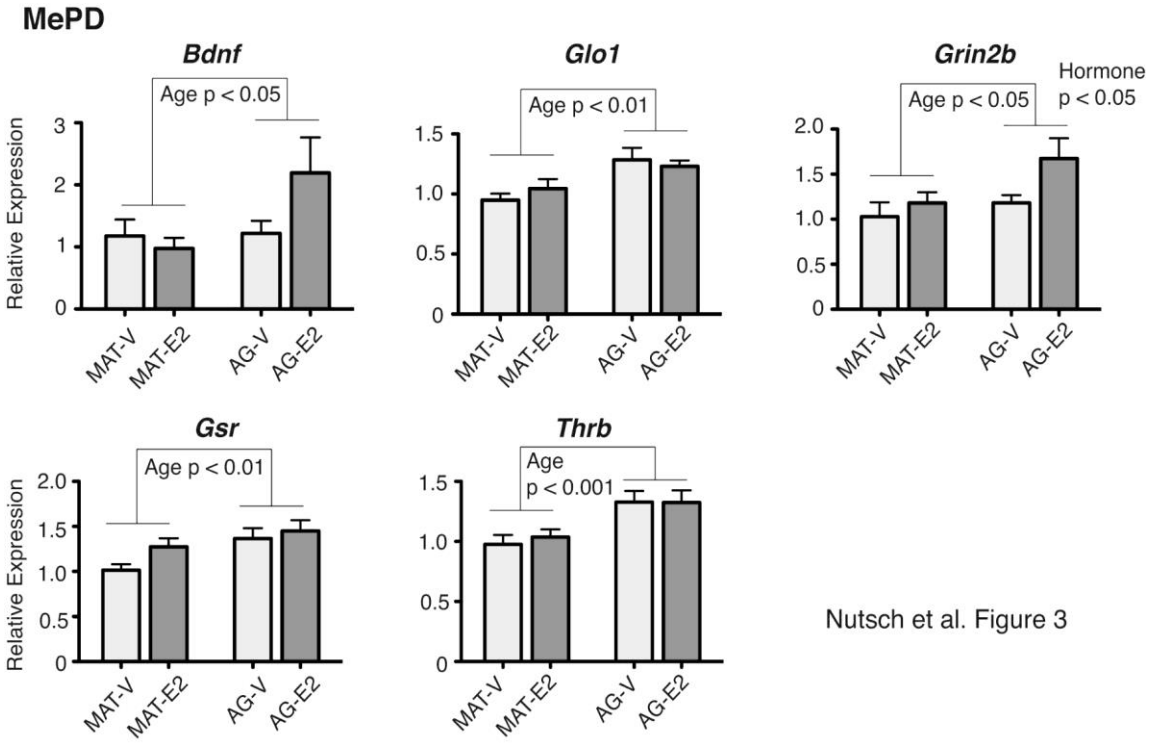

Figure 4.6 Effects of age and hormone in the MePD.

Genes with significant main effects of age or hormone, but no interactions, are shown for the MePD. Five genes showed increased expression in aged compared to mature animals: *Glo1, Bdnf, Gsr*, *Thrb,* and *Grin2b. Grin2b* also had a significant hormone effect, and was higher in E2 than Vehicle males ($p < 0.05$). P-values for significant main effects are shown.

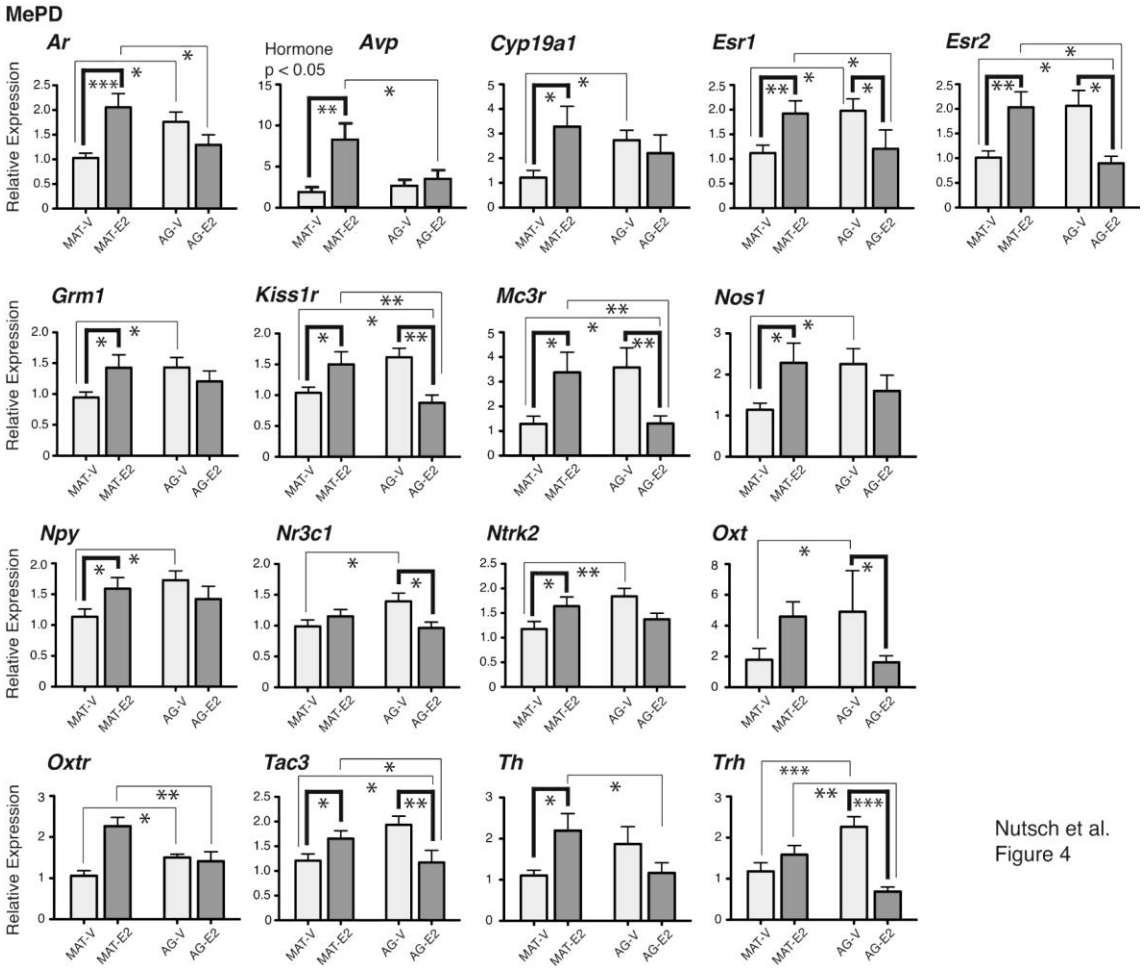

Figure 4.7 Interactions in the MePD.

Genes with significant interactions between age and hormone treatment are shown for the MePD. Significant effects of hormone within an age group are indicated by bold brackets. Significant effects of age within a hormone group are indicated by thin brackets. P-values are indicated as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

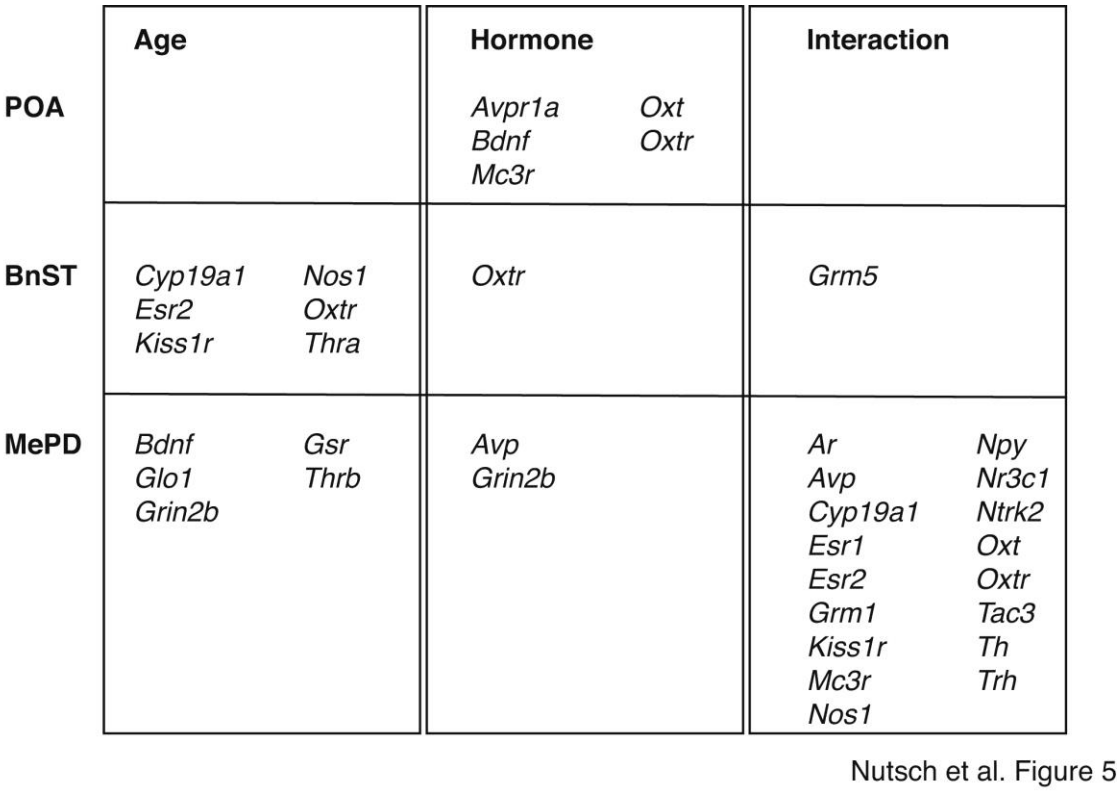

Figure 4.8 A summary and comparison of the changes in the POA, BnST and MePD.

Summary of genes with significant differences in the POA, BnST and MePD due to Age (left), Hormone (right) or with a significant interaction (center), and corresponding p-values.

MePD results for non-significant genes

	MAT-Vehicle		MAT-E2		AG-Vehicle		AG-E2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Avpr1a	1.03	0.11	0.96	0.09	1.40	0.23	1.03	0.13
Bdnf	1.18	0.26	0.98	0.17	1.22	0.20	2.19	0.57
Crh	1.28	0.30	2.03	0.36	1.44	0.28	1.06	0.35
Dbh	0.98	0.12	2.48	0.53	0.82	0.05	1.43	0.35
Drd1a	1.22	0.23	1.62	0.28	1.36	0.11	1.35	0.40
Drd2	1.14	0.27	0.95	0.16	0.85	0.10	1.07	0.44
Drd3	0.97	0.16	1.07	0.29	0.97	0.22	0.60	0.10
Esr2	1.01	0.14	2.03	0.31	2.06	0.31	0.90	0.13
Grm1	0.94	0.09	1.43	0.21	1.43	0.16	1.21	0.17
Gper	1.18	0.20	1.45	0.21	1.60	0.21	1.07	0.10
Grm5	0.96	0.10	1.15	0.12	1.02	0.07	1.16	0.11
Gsr	1.01	0.06	1.28	0.09	1.49	0.16	1.45	0.12
Htr1a	1.26	0.24	1.69	0.26	1.70	0.13	1.69	0.41
Kiss1	1.17	0.19	11.87	5.93	3.77	0.97	5.19	2.18
Lepr	0.86	0.08	0.92	0.08	1.15	0.17	1.25	0.26
Mc3r	1.29	0.31	3.38	0.82	3.58	0.80	1.31	0.31
Mc4r	1.01	0.20	1.20	0.16	0.91	0.10	1.11	0.27
Mc5r	1.16	0.24	1.46	0.30	1.45	0.27	1.51	0.36
Ncoa2	1.08	0.10	1.23	0.10	1.47	0.12	1.21	0.13
Nos1	1.14	0.16	2.28	0.48	2.26	0.37	1.60	0.38
Npvf	1.13	0.30	1.43	0.27	1.26	0.25	2.65	1.42
Pdyn	0.98	0.21	1.44	0.27	0.90	0.13	1.15	0.26
Pgr	1.19	0.16	1.51	0.17	1.46	0.17	1.41	0.12
Pomc	1.13	0.24	0.87	0.13	1.47	0.45	1.05	0.60
Slc6a3	1.40	0.54	1.77	0.60	3.79	2.54	1.53	0.45
Slc6a4	1.05	0.15	1.25	0.27	1.47	0.55	1.07	0.32
Tac2	1.09	0.28	1.36	0.25	1.16	0.19	0.81	0.20
Thra	1.07	0.11	1.36	0.12	1.33	0.10	1.22	0.10
Th	1.10	0.13	2.20	0.41	1.87	0.40	1.17	0.25

Figure 4.9 Supplemental Table 4

Expression of non-significant genes in the MePD. Relative expression (mean + SEM) is shown for those genes not significantly affected by E2, age, or their interaction. N/D = Not detectable (did not amplify).

4.4.3 Effects of Estradiol and Aging on Serum Hormone Concentrations

Eight of the serum hormones were significantly affected by treatment and/or aging (BDNF, E2, FSH, GH, LH, PRL, T4, and TSH; Figure 4.10). Serum E2 was measured to validate the efficacy of the hormone treatment regime. Significant main effects of both age and E2 treatment were found, with E2 concentrations higher ($p < 0.01$) in the E2 replacement groups, as expected. Concentrations of E2 were also elevated in aged compared to young animals ($p < 0.05$). Both serum LH and TSH had a main treatment effects, and were lower in E2 than Vehicle rats ($p < 0.001$, $p < 0.05$, respectively). Serum FSH showed main effects for both age and E2, and was decreased significantly for both ($p < 0.01$ for both). GH concentrations decreased with age ($p < 0.01$) and increased with E2 treatment ($p < 0.001$). Significant interactions of age and hormone were found for BDNF, PRL and T4

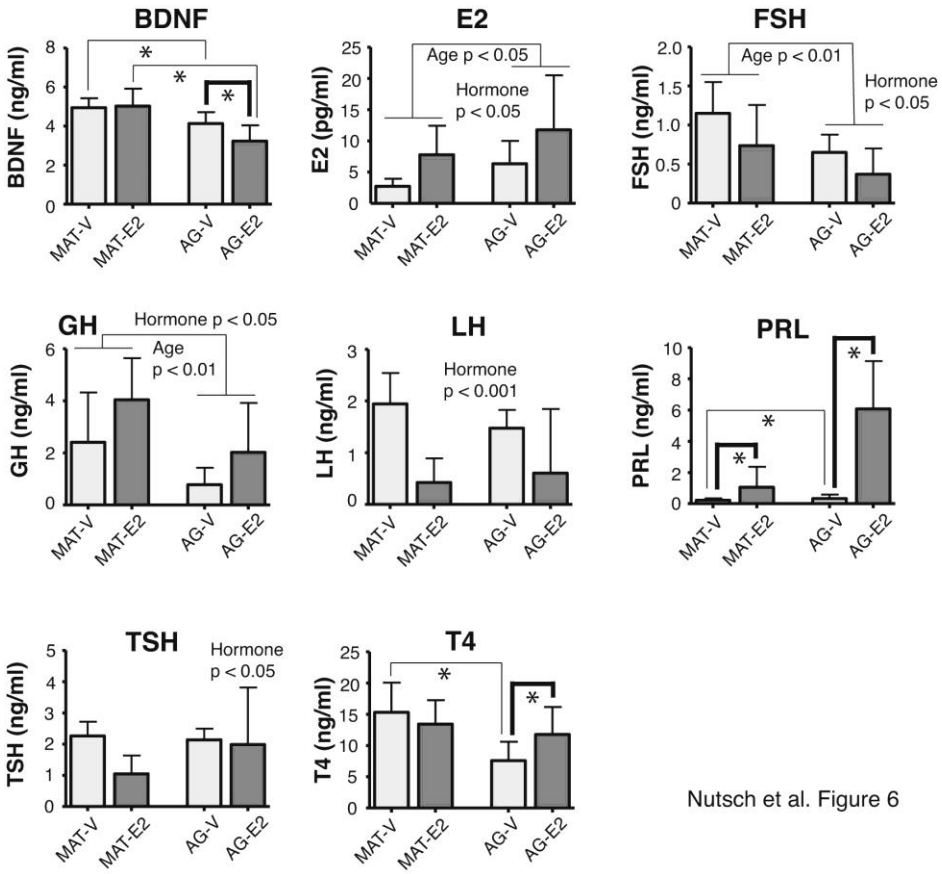

Figure 4.10 Serum hormones affected by hormone treatment.

Serum hormones are shown that were affected by hormone treatment (LH, TSH), main effects of both age and hormone (E2, FSH, GH), and those with significant interactions (BDNF, PRL, T4). LH and TSH were significantly decreased in E2 compared to Vehicle males. Three serum hormones had main effects of both age and hormone: E2, FSH and GH, with p-values shown for the age effects; the hormone treatment effects were all $p < 0.05$. Three serum hormones, BDNF, PRL, and T4, had an interaction of age and hormone treatment. Significant effects of hormone within an age group are indicated by bold brackets. Significant effects of age within a hormone group are indicated by thin brackets. Interactions p-values are indicated as: *, $p < 0.05$.

4.4.3 Heatmaps

To ascertain relationships among groups of genes within each region, and to determine clustering of animals by age and treatment, we conducted a hierarchical cluster analysis and generated heatmaps (Figure 4.11). An unbiased group order was generated based on similarities of gene groups and the ordering of the treatment groups; these differed for the three regions in accordance with differential effects of age, hormones, and their interactions. Five to six groups were generated within each region based on top-down hierarchical splitting, so that each group would contain at least 5% of the total genes (Yin et al., 2015).

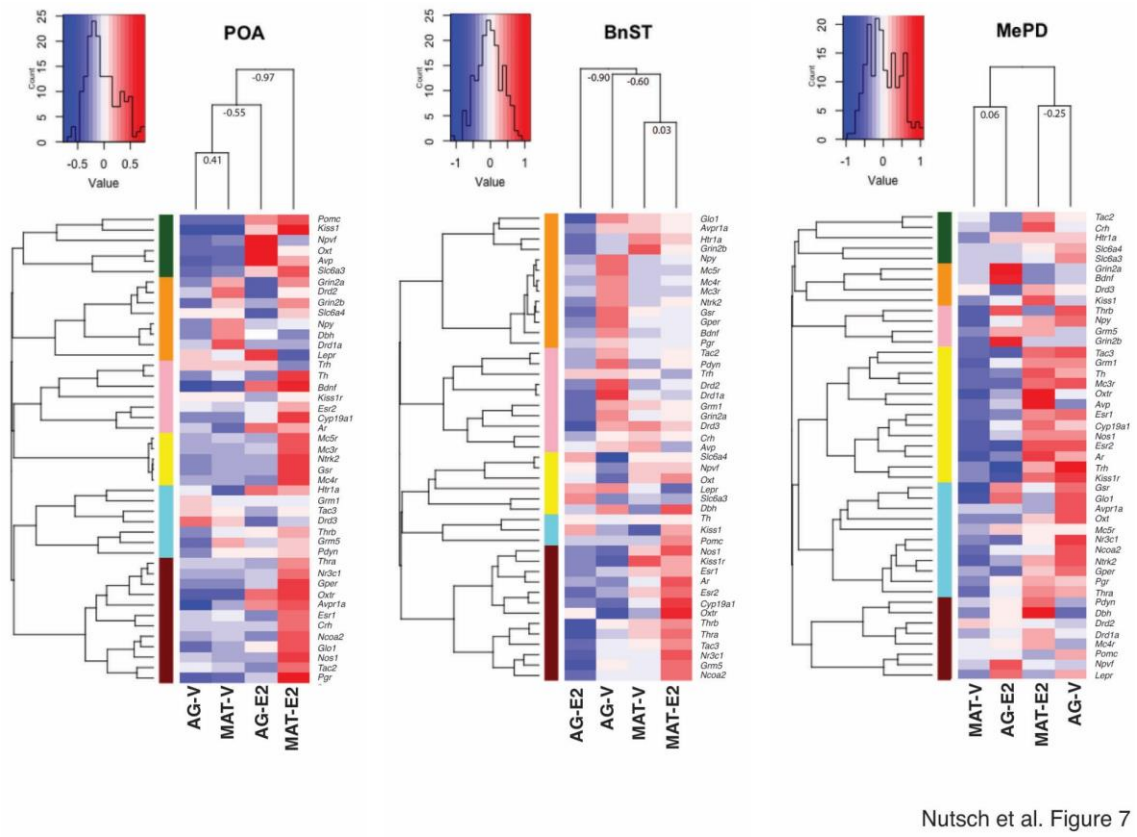

Figure 4.11 Heatmaps of gene expression in the BnST, POA and MePD are shown..

Five to six groups were generated within each region based on top-down hierarchical splitting, indicated by color codes, so that each group would contain at least 5% of the total gene.

4.5 DISCUSSION

This study sought to evaluate the effects of estradiol, aging, and their interactions in male rats, focusing on gene expression in the POA, BnST, and MePD, and peripheral serum hormone levels. These regions were selected because of their high concentration of steroid hormone receptors, as well as the high expression of aromatase, the enzyme that converts testosterone into estradiol (Mitra et al., 2003; Perez et al., 2003; Takahashi et al., 2006; Stanic et al., 2014). As part of the brain's social decision-making network, these regions are highly conserved across species (O'Connell and Hofmann, 2012) and play critical roles in a variety of reproductive, social, olfactory, and affective behaviors and underlying physiology. The POA contains GnRH and kisspeptin neurons that are obligatory to reproductive function (Xue et al., 2014; d'Anglemont de Tassigny, 2009), and kisspeptin is necessary for the mediation of steroid hormone feedback actions on the hypothalamus (Smith et al., 2013; Clarke et al., 2015). The BnST, along with the MePD, is involved in olfaction and social recognition (Ervin et al., 2015). It is also sexually dimorphic, with males having a larger BnST than females (Bian et al., 2014). The POA, BnST and MePD are all modulated by estradiol, mediated primarily by ERα. Of these, the POA and BnST have larger roles in social preference, mating and territorial aggression, while the MePD is linked to social recognition (Russell et al., 2012; Ervin et al., 2015; Sano et al., 2013). Other studies have linked the MePD with aggressive behaviors (Unger et al., 2015; Sano et al., 2016).

4.5.1 Estradiol but not age increases expression of five neuroendocrine genes in the POA

One of our most surprising findings was that in the POA, only 5 of the 46 genes (*Avpr1a, Bdnf, Mc3r, Oxt, Oxtr)* showed significant changes, with expression increased in the E2 treatment groups but no effect of age. Notably, three of these genes encoding oxytocin, oxytocin receptor, and vasopressin receptor, are critical to the control of social behavior, although most of that literature has focused on the paraventricular nucleus and supraoptic nucleus (Winslow and Insel, 2004; Hrabovszky et al., 2004). However, little is known about E2 regulation of these nonapeptide signaling systems in the POA. It was reported in females that E2 tended to upregulate oxytocin mRNA expression in the POA, but this was not significant (Chung et al., 1991). Oxytocin in the POA facilitates sexual behavior in males, and this undergoes age-related impairments (Gil et al., 2011). Furthermore, oxytocin colocalizes with the p450 aromatase enzyme in the male POA (El-Emam et al., 2013), suggesting the capacity of these cells to convert testosterone to estradiol. Regarding BDNF, to our knowledge age- and estrogen-induced changes in the POA have not been reported in males, but other brain regions have been examined. Katoh-Semba et al. (1998) reported age-related increases of BDNF in the hippocampus and cortex of male mice. There is also strong evidence for estradiol regulation of BDNF in the female brain, including in hypothalamic regions (Zhu et al., 2013). While sex differences in expression of BNDF were reported in hippocampus (Franklin & Perrot-Sinal, 2006), this was not found in the hypothalamus of mice (Ren-Patterson et al., 2006)

4.5.2 The BnST is predominately affected by age

The BnST, unlike the POA, showed mainly age-related changes, with most identified genes expressed at lower levels in aged than young males. The BnST is involved in social recognition, stress responses, duration of fear states, and mating

behavior, and projects more directly to areas important in activation of the HPA axis than areas in the amygdala (Lebow and Chen, 2016). Though no studies were found for the BnST, in the PVN E2 acts through ERbeta to increase NOS, thereby increasing NO production (Gingerich and Krukoff, 2005; Gingerich and Krukoff, 2006). Decreased oxytocin receptor (OTR) expression and binding in the BnST seems to be associated with decreased aggression. Rats who were first time fathers exhibited facilitated parental care compared to virgins, and lower OTR expression in the BnST, but not the MeA or POA (Perea-Rodriguez, 2015). On the other hand, when introduced to a novel male intruder, rats classified as excessively aggressive also showed the highest levels of OTR binding in the BnST (Calcagnoli et al., 2014).

4.5.3 Genes in the MePD were affected by interactions of age and E2 treatment

Sixteen genes in the MePD, falling into several functional classes including nuclear hormone receptors, neurotransmitters and neuropeptides and their receptors, had significant interaction effects. The MePD is involved in both olfaction and social behavior, and receive projections from the medial amygdala (MeA), the latter conveying chemosensory information (Maras and Petrulis, 2006; Maras and Petrulis, 2010) but the former having higher density of steroid hormone receptors (Maras and Petrulis, 2010). The MePD itself makes reciprocal projections with areas involved in reproductive behavior, including the POA and BnST (Pardo-Bellver, 2012; Morrell et al., 1984). Either castration or lesions of the MePD resulted in decreased preference for female odors by males (Maras and Petrulis, 2006; Xiao et al., 2015). Interestingly, OTR binding densities in the medial amygdala correlated positively with social interest in males, but

negatively in females, and males also show higher overall levels of OTR binding than females (Dumais et al., 2013).

It is notable that many affected genes in the MePD showed a pattern by which E2 increased expression in mature animals, but had no effect or decreased expression in older animals. For those genes that were up-regulated by E2 in mature males and were unaffected in aged males (*Nos1, Th, Cyp19a1, Grm1, Npy, Ntrk2*), this result is consistent with a loss of E2-responsiveness with aging. By contrast, genes that were up-regulated by E2 in mature males and down-regulated by E2 in aging males (*Esr1, Esr2, Mc3r, Tac3, Kiss1r*), continue to responsive to this hormone, and the expression pattern may represent a compensatory mechanism caused by E2 treatment with aging. For these latter genes, castrated males had higher gene expression with aging, and E2 treatment in the aging animals "normalized" expression to the mature castrate baseline.

4.5.4 Serum hormones change as a result of age and estradiol

A hallmark of aging is changes in concentrations of the release patterns of peripheral hormones (Gore, 1998). Our results add to the literature of effects of castration and E2 treatment in the context of aging in male rats. Interestingly, while E2 treatment was effective in increasing serum E2 concentrations, aged males, whether given vehicle or E2, had higher serum E2 concentrations than their younger counterparts. These aged males are significantly heavier and have much larger fat depots, so this difference may be due to peripheral aromatization of adrenal hormones. The interpretation of our gene and hormone data therefore needs to be made in this context.

We found that serum FSH and GH underwent significant decreases with age, and a significant interaction of age and hormone on BDNF driven by lower concentrations in the aged group. These results are largely consistent with the literature in rats and men (Raven et al., 2011, Heaton et al., 2003, Dobado-Berries et al., 1996; Driscoll et al., 2012; Lommatzsch et al., 2005). For FSH, we found an age-related decrease as well as a down-regulation with E2 treatment, the latter expected based on negative feedback effects of E2 on gonadotropins in humans of both sexes (Raven et al., 2011, Greenblatt et al., 1976) as well as female rats (Yin et al., 2015).

We did not see changes in LH in older animals, a finding that differs from previous studies that reported lower LH during aging in male rats and men (Bonavera et al., 1997; Veldhuis et al., 2005; Walker et al., 2013); however, that published work was conducted in intact males, and one study did show an increase in LH with age (Greenblatt et al., 1976). Two points must be considered for these LH results; first, we did not take multiple measures of serum hormones in order to evaluate changes in pulsatility or diurnal rhythms, which undergo age-related changes. Moreover, serum LH decreased in response to estradiol, consistent with negative feedback actions, and our data show that this response is maintained even in our aging male rats, similar to what has been reported for aging men (Veldhuis and Dufau, 1993) and female rats (Yin et al., 2015). The serum PRL results were very interesting, as they showed a profound age-related increase, together with an up-regulation by E2 that was much greater in the old than the young male rats. The age-related increase in prolactin has been reported previously in intact

male rats (Estes & Simpkins, 1980), but to our knowledge, this change in sensitivity to E2 with age is a novel finding, and differs from our aging female rats whose LH response to E2 treatment was comparable in young and aging animals (Yin et al., 2015).

Finally, the thyroid hormone axis hormones also showed regulation by age and hormone. Serum T4 was lowest in aged-vehicle animals and significantly different from the mature-vehicle and aged-E2 males. Serum TSH was decreased in the E2 groups. These results are quite different to what we reported in ovariectomized females, in which TSH was increased by E2 (the opposite of our castrated males in the current study), and T4 was decreased by E2 at all ages in the females but there was no effect of age (Yin et al., 2015). Further work on regulation of the hypothalamic-pituitary-thyroid axis by E2 is merited

4.5.5 Conclusion

Our study demonstrates that E2 regulation of gene expression is region specific and changes during aging. In particular, our results for age-related changes and estradiol regulation in the BnST and MePD suggest that these are important regions for future research on functional outcomes of how estradiol influences the male brain during aging. The gene expression results may inform important targets for further analysis, especially, to determine if the protein products change in a similar manner, and consequences on other behavioral or physiological endpoints not studied herein. Clearly, the efficacy of hormone treatments are profoundly influenced by chronological age, and it is important to appreciate that the aging brain continues to be very responsive to E2 treatment, albeit differentially from the young adult brain).

Chapter 5: Microdialysis

5.1 Abstract

Androgens and estrogens both act to facilitate sexual behavior in males. The mPOA is a critical region involved in the hormonal control of masculine sexual behavior, particularly its facilitation by estradiol. Estradiol may be acting by contributing to increased dopamine release in the MPOA during copulation, which is also necessary for male sexual behavior. As males age they show impairments in sexual behavior, which is not reversed by administration of exogenous hormones. However, only males with decreased dopamine in the MPOA show behavioral impairments; those with normal dopamine levels continue to copulate. We hypothesized that there were age-related impairments in estradiol's ability to maintain dopamine in the MPOA, and that this accounted for the observed impairments in behavior in older animals. We tested this by measuring dopamine release during olfactory exposure to a female and copulation in mature (5 mo), middle-aged (12 mo) and aged (18 mo) castrated male rats given estradiol or vehicle replacement. Unfortunately due to technical difficulties we had very small n's for some of our groups, and were not able to determine if our observed lack of statistical significance between groups was because there is in fact no difference in dopamine release between young, middle-aged and aged animals, or if we didn't have the power to detect any differences that were there.

5.2 Introduction

In males the medial preoptic area (MPOA) is known to be essential for sexual behavior. Electrical stimulation of the MPOA facilitates both male sexual behavior and

erectile function, while lesions of the MPOA significantly impair all measures of sexual behavior (reviewed in Hull and Dominguez, 2007). Both dopamine and gonadal steroid hormones, such as estradiol and testosterone, facilitate sexual behavior, at least partially through interactions with the MPOA (Roselli et al. 2003, Russell 2012, Putnam et al. 2003). Aged males show impairments in sexual behavior, even when given testosterone replacement, indicating age-related impairments in neuroendocrine systems underlying copulation (Smith et al. 1992, Chambers and Phoenix, 1984, Wu and Gore 2010).

Male sexual behavior is hormone dependent. Castrated animals fail to copulate, while hormone replacement with testosterone restores sexual behavior to levels similar to those seen in castrates (Putnam et al. 2003, McGinnis et al. 1989). Estradiol, a metabolite of testosterone, also facilitates sexual behavior in castrates, while animals given 5alpha-dihydrotestosterone (DHT), which cannot be aromatized to estradiol and only binds androgen receptors, fails to stimulate male sexual behavior (McGinnis et al. 1989, Putnam et al. 2003). Estrogen receptors are expressed in many areas of the brain associated with male sexual behavior, but activation of ERalpha in the MPOA has been shown be necessary and sufficient to activate sexual behavior in males (Russell et al. 2012). Age-related impairments in behavior do not seem to be merely a matter of changes in serum hormones levels, as estradiol has been reported to increase, decrease, or remain the same in males during aging (Luine et al. 2007, Herath et al. 2001, Wu and Gore 2009, Goya et al. 1990, Smith et al. 1992, Herrera-Perez et al. 2008), and serum estradiol levels do not correlate with male sexual behavior (Smith et al. 1992, Wu and Gore 2010).

One way estradiol may facilitate male sexual behavior is by increasing the release of dopamine in MPOA. Dopamine in the MPOA is important for coordinating sensory input and sexual motivation with motor output and genital function (Hull et al. 1995, Hull et al. 2004, Dominguez and Hull 2005). Extracellular dopamine increases in the MPOA during exposure to estrous females and during copulation (Hull et al. 1995). In addition, estradiol is responsible for maintaining basal extracellular dopamine in castrates (Putnam et al. 2003), as well as increasing the expression of nitric oxide synthase (NOS), an enzyme essential for glutamate induced dopamine release in the MPOA (Putnam et al. 2005, Dominguez et al. 2004, Dominguez and Hull 2005). Aged males that maintain mounts and intromissions have MPOA dopamine concentrations equivalent to young animals, indicating the aging brain is still able to respond to dopamine, and the problem is with deficits in dopamine expression and release (Chen et al. 2007).

Given estradiol's importance for sexual behavior and extracellular dopamine in the MPOA, we hypothesized that age-related impairments in sexual behavior were due to a diminished ability of estradiol to facilitate dopamine release during sexual behavior in aged males. In order to test this hypothesis we measured dopamine during copulation in the MPOA of young (5 mo), middle-aged (12 mo) and aged castrates given estradiol replacement.

5.3 MATERIALS AND METHODS

5.3.1 Subjects

Sprague-Dawley male rats (Harlan, Indianapolis, IN; 3 months (n = 20), 10 months (n=16) or 12 months (n=24) at arrival) were pair housed in large plastic cages, in a climate-controlled room, on a reverse light/dark cycle (12 hours light/12 hours dark; lights off at 10 a.m.). Food and water were freely available. Conspecific females (n = 16) were purchased as young adults, and ovariectomized under ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (4 mg/kg) anesthesia. They were brought into behavioral estrus with an injection of 4 μg estradiol benzoate (s.c.), followed 44 hours later by an injection of 400 μg (s.c.) progesterone. Testing took place 4 hours later. Sexual receptivity of females was confirmed by placing her into a cage with a separate stud male shortly before the test began and watching for lordosis. All procedures were done in accordance with the National Institutes of Health's Guidelines for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Middle-Aged (MA) and aged males (AG, 12 months) were retired breeders at purchase and were not given further experience sessions in the lab. Mature males (MAT, 3 months) were given sexual experience in lab in order match experience levels with AG males. MAT animals were allowed to mate with a sexually receptive female for 90 min, every other day, for 14 days, for a total of 7 experience sessions. On an 8th day animals

were observed to confirm that they achieved at least two ejaculations during the final experience session. All of the males met these criteria and were included in the study.

5.3.2 Surgeries

Castration surgery was conducted under isoflurane anesthesia when rats were 3.5, 10, or 16 months of age. One month later, at 4.5, 11 or 17 months, males were implanted with 12 mm Silastic capsules (1.98 I.D. x 3.18 O.D.) containing either estradiol (E, 5% in cholesterol), or cholesterol (C, 100% cholesterol). Two weeks after hormone implantation, all rats were surgically implanted with intracranial guide cannulae (MAB 4.15.IC, SciPro Inc., Sanborn, NY, USA), lowered to 1mm above the MPAO (AP, -0.3 mm; ML, +3.1 mm; DV, -8.0 mm). The surgery was performed under isoflurane anesthesia (Paxinos and Watson 1998). Three small screws were inserted in the skull and then cannulas were fixed in place with dental resin (Hygenic Corporation, Cuyahoga Falls, OH).

5.3.3 Behavioral Testing

After 14 days of recovery from surgery, a probe (MAB 4.15.2.PES, SciPro Inc., Sanborn NY, USA) was inserted into the MPAO while animals were under light sedation with isoflurane anesthesia. Filtered and degassed Dulbecco's PBS (138 mM NaCl, 2.1 mM KCl, 0.5 mM MgCl2, 1.5 mM KH2PO4, 8.1 mm NaHPO4, 1.2 mm CaCl2, and 0.5 mM D-glucose, pH 7.4; Sigma Aldrich, St. Louis, MO) was perfused through the probe at a rate of 1 μL/min using a 1mL gas-tight syringe on an infusion pump (PHD 22, Harvard Apparatus, Holliston, MA). Animals were allowed to acclimate to the probe insertion in the testing chamber for 4 hours prior to sample collection. Food and water

were available during this time period and removed once sample collection began. During testing the flow rate was increased to 2 μL/min, and dialysate samples were collected every 12 min. After 36 min of collecting baseline samples, a novel female was introduced in a small mesh cage, allowing olfactory exposure but preventing full access to the female and copulation. After 24 min of olfactory exposure to the females, the mesh cage was removed, the female was placed in the males's cage, and the animals were allowed to copulate. After 30 min the female was removed and 2 post copulatory samples were collected (24 min). After collection, samples were frozen at −80°C until analyzed.

5.3.6 Tissue Collection and Probe Placement

Immediately following microdialysis, subjects were euthanized with a lethal dose of Euthasol (0.3 mL/animal; Virbac Animal Health, Inc.; Fort Worth, TX). Brains were then extracted and post-fixed in 4% PFA for 48 hours at 4°C before being transferred to a 30% sucrose solution and stored at 4°C for at least 48 hrs until sectioning. Coronal sections were cut at 35 μm at the level of the MPOA and were stored at -20°C in a cryoprotectant solution. Tissue was then mounted onto slides, washed in 0.1M PB, stained with methyl green, dehydrated, and coverslipped to visualize placement.

5.3.7 High Performance Liquid Chromatography with Electrochemical Detection

HPLC-EC was used to determine the levels of DA present in the MPOA following olfactory exposure to a female or copulation. Samples were individually thawed and then injected into a 6μL loop. Monoamines were separated by an Acclaim PA2 reversed phase column (2.1 X 100mm, 2.2 μm packing) and detected using an Antec VT-03 electrochemical flow cell with ISAAC reference electrode and a working potential

of 550mV at 35ºC. The mobile phase consisted of 12.5% MeOH, 50 mM phosphoric acid, 8 mM NaCl, 0.1 mM EDTA, and 150 mg/L octanesulfonic acid, with a pH of 5.6, and was pumped at a rate of 0.225 mL/min. Chromatography was performed using an Antec Decade 2 microelectrochemical detector attached to a PC running Clarity chromatography software (Data Apex, Prague, Czech Republic).

5.3.7 Data Analysis

Dopamine was analyzed as the percent change from the average of an animal's three baseline samples. A Grubb's test for outliers was conducted, and up to two outliers per group were removed if indicated. A three-way analysis of variance was performed to test for main effects of age, hormones, and interactions, followed by a Tukey post-hoc analysis, when indicated. Data failed tests for both normality and homogeneity, even when transformed, so untransformed values were used and p-values are reported with the acknowledgement that these assumptions were not met. Data analyses were performed with Sigma Stat 7.

5.4 RESULTS

4.4.1 Dopamine Release in the POA

A three-way ANOVA using age, hormone, and time as factors revealed there was an effect of time, such that C2 was higher than BL3 ($F=2.81$, $p < 0.01$). There was no effect of age ($F=0.21$, $p = 0.542$), or hormone ($F = 3.14$, $p = 0.077$), or an age x hormone x time interaction ($F=0.83$, $p=0.576$).

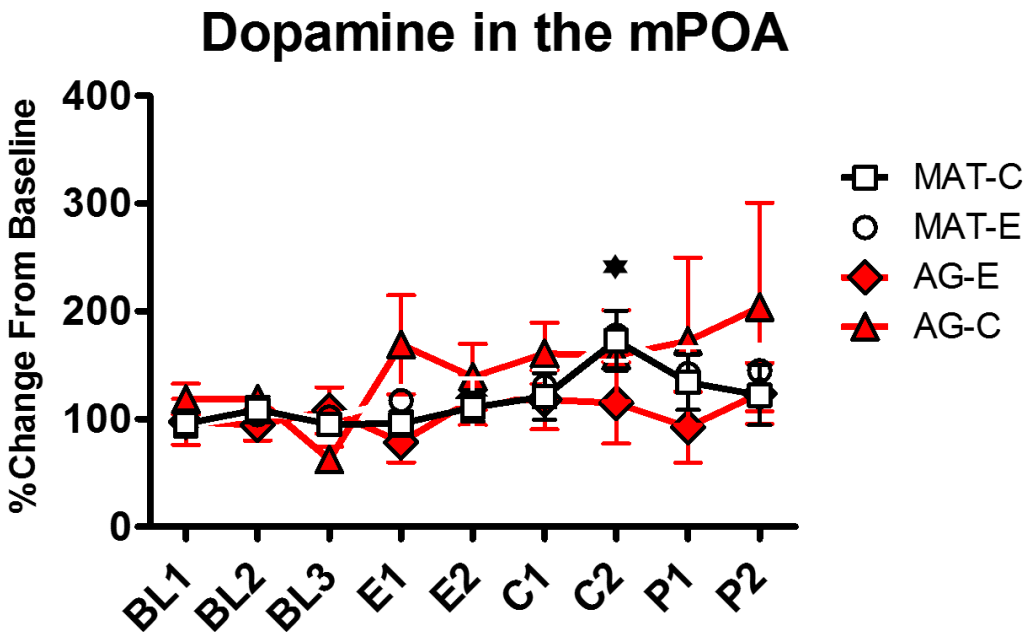

Figure 5.1 .Dopamine in the mPOA during exposure to females in matured and aged males

Dopamine release changes in percent from baseline between MAT-C (*n*=11), MAT-E (n=16), AG-C (n=4) and AG-E (*n*=7) male rats. There was an effect of time, with C2 being significantly higher than BL3 ($F=2.81$, $p < 0.01$). There were no significant interactions between age x time, hormone x time or age x hormone x time. Values are expressed as mean ± SEM.

5.5 DISCUSSION

I did not find any significant differences due to age or estradiol administration in dopamine's % change from baseline in the mPOA during exposure to a female. There was an increase in dopamine at C2, but only when compared to BL3, not BL1 or BL2, and only when all four groups were averaged together. I suspect that this difference was only a result of the small group sizes in the aged animals, and not something specific to either BL3 or C2. There does appear to be a trend for increased dopamine release in AG-C compared to AG-E, but again, it is difficult to tell with so few animals. Interpretation of the data is also more difficult without a positive control group, or a way to confirm that any dopamine increase that was observed was not due to tissue damage from inserting the probe. I think there would likely be some age-related changes if the study were repeated with larger groups sizes and additional control groups.

There were several factors that made completion of this study difficult. One is in obtaining aged animals. There are limited numbers of retired breeders available, with anywhere 9-12 months being the oldest animals Harlan offers. This means animals for the AG group must be purchased a minimum of 4 months before beginning testing, and it is very difficult to add any animals if needed. Older animals also had more difficulties recovering from surgeries than young animals. Implanting the cannula took close to an hour, and almost half of the aged animals never woke up after surgery. All of the age groups also had at least one animal who knocked off their head cap in the period between the final surgery and behavioral testing.

I also ran into some unexpected problems measuring dopamine with the HPLC. There was something in dialysate that produced at spike at the same time point that dopamine would have, and prevented me from measuring the actual dopamine concentration. This problem was fixed, but some groups, particularly MA-C (n=1), MA-E (n=1) and AG-C (n=4), ended up with very few remaining animals. In addition to small group sizes within groups with more than one there was a high level of variability.

Many of the animals, especially in the two older groups, also failed to take much of an interest in the females. Though previous studies have found ejaculation to be relatively rare in females only given estradiol replacement, they have found that males will investigate the females, mount and intromit. Increased dopamine concentrations in the mPOA during copulation are a result of not only sensory information, such as hearing and smelling her, but genitosensory input from interacting with the female. The lack of interactions between the males and the females during testing likely contributed to my not seeing a rise in dopamine during the testing session.

Chapter 6: General discussion

6.1 Summary of results

The results presented here demonstrate that it is important to consider the effects of estradiol in males, and that the effects of estradiol may differ with age. I found age-related changes in correlations between serum estradiol and mount and intromission latencies in males, as well as changes in the way that gene expression responds to estradiol administration. My findings agree with previous reports showing that estradiol influences sexual motivation in males (Roselli et al. 2003, Cross and Roselli 1999, Hamson et al. 2009), and but show interactions between estradiol and age on mRNA expression that emphasize that studies done in young animals do not necessarily generalize to aged ones, and regions important for the expression of sexual behavior undergo different age-related changes in response to steroid hormones (Figure 6.1). I also found additional evidence that experience plays a role in modulating enzymes regulated by estradiol that are important for sexual behavior (Figure 6.2) (Dominguez and Hull, 2005). Taken together, these results demonstrate that estradiol effects both the brain and behavior in males, and needs to be considered when examining aging neuroendocrine systems.

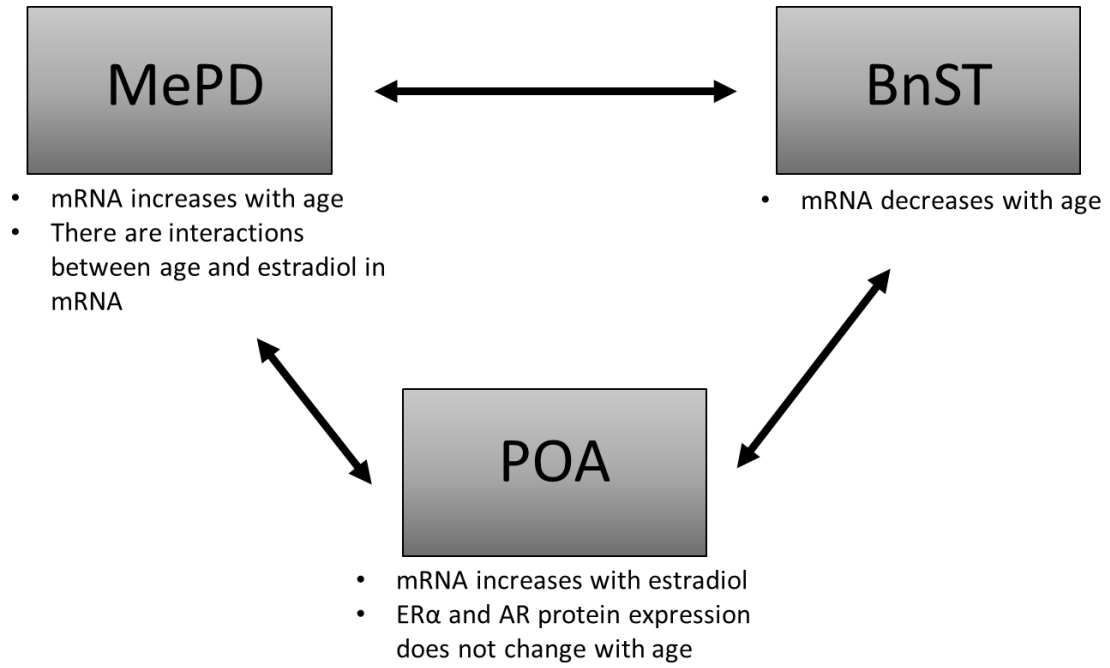

Figure 6.1 Model of MPOA changes with age and estradiol in males.

The POA, MePD and BnST exhibit different patterns of mRNA expression during aging. In the MePD genes with main effects of age had increased mRNA expression in aged groups, while many more genes showed an interaction between age and estradiol on expression. In the BnST mRNA expression increased in aged groups. In the POA expression increased with estradiol, but there were no age-related changes. There were also no age-related changes in ERα or AR protein expression in the POA..

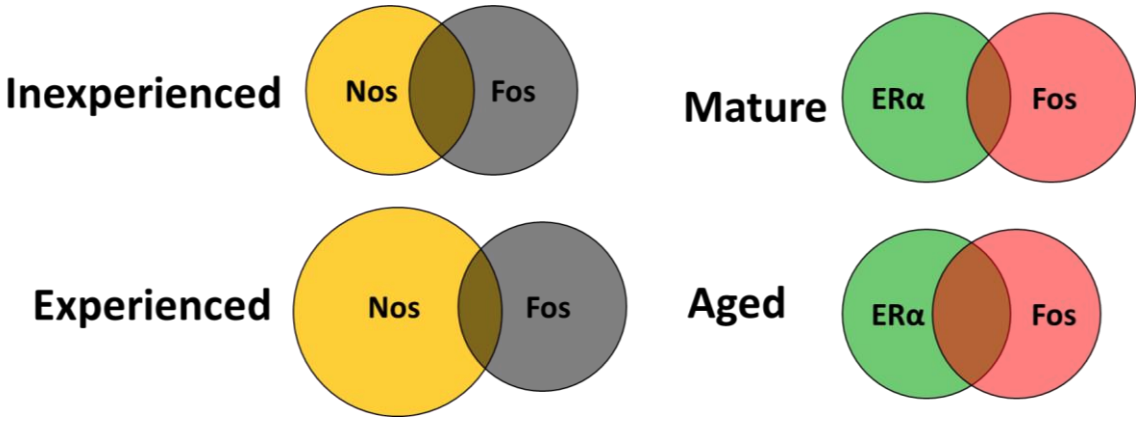

Figure 6.2 Model of MPOA changes with age and sexual experience in males.

NOS is increased in sexually experienced males, but the percent of NOS cells activated by mating, indicated by a colocalization with Fos, is higher in inexperienced males mating for the first time. The percent of ERα cells activated by sexual behavior increased in mature vs. aged males. Neither the number of ERα- or the number of Fos-positive cells changed as a result age..

6.2 Sexual Experience and NOS

Evidence suggest that activation of NOS plays a key role in the both the expression of sexual behavior, and the experience related facilitation of sexual behavior (Hull and Domingeuz 2006, Hull and Domingeuz 2007). Estradiol regulates NOS both by facilitating activation of NOS by glutamate and NMDA receptors, and maintaining NOS expression (Hull and Domingeuz 2006, Putnam et al 2005, d'Anglemont de Tassigny et al. 2009). Increased nitric oxide (NO) in the MPOA is associated with increased extracellular dopamine in the MPOA, which is necessary for copulation (reviewed in Hull and Dominguez, 2006). One of the ways estradiol may be facilitating dopamine release in the POA during copulation is through increasing NO production (Putnam et al. 2003, Putnam et al. 2005). A knock down of ERalpha in the MPOA of intact male mice reduced NOS expression, indicating that both NOS expression and male sexual behavior are dependent on ERalpha in the MPOA (Sano et al. 2013, Russell et al. 2012). In aged males, inducible NOS was increased, but neuronal NOS was not (Wang et al. 2002). More studies will be needed to define the role of estradiol in nitric oxide mediated dopamine release and sexual behavior in aged males.

6.3 Serum Hormone Production in Aging Males

Steroid hormones are necessary for copulation, but age related impairments in male sexual behavior do not seem to be due to a lack of the ability to produce steroid hormones. In aged males, the testes are still able to produce testosterone and estradiol in response to increased GnRH and LH, despite some deficits in the aging HPG axis (Liu et

al. 2005, Iranmanesh 2010, Gruenewald 1992, Veldhuis 2005, Keenan 2011), and though serum testosterone and estradiol often decrease with age (Greenblatt 1976, Smith et al 1992, Wu and Gore 2009, Leifke 200, Gruenewald 2000), this is not always the case (Wu and Gore 2009, Lakshman et al. 2010, Herath et al. 2001, Greenblatt et al. 1976). Even if there is a reduction in serum hormone concentrations in aged animals, hormone concentrations cannot be used to predict impairments in sexual behavior, and hormone replacement does not eliminate age-related impairments (Chambers and Phoenix 1986, Smith et al. 1992, Wu and Gore 2010).

Estradiol can also be temporarily produced locally in in some regions by activation of aromatase (Reviewed in Balthazart et al. 2002), an enzyme that is highly expressed in the MeA, BnST and POA in males (Wagner 1996, Stanic et al. 2014, Tabatdze et al. 2014). In the MPOA aromatase is decreased by castration, and its increase after testosterone replacement is AR-dependent, a mechanism which appears to be specific to the MPOA (Roselli et al. 1997, Resko et al. 2000). While DHT alone increases aromatase expression in castrates, it was not as effective as either testosterone or DHT and estradiol combined; estradiol alone did not increase aromatase (Roselli et al. 1991). The exact mechanism behind the increase of aromatase in animals given estradiol and DHT is not known, but it has been found that AR occupation by DHT is increased in the presence of estradiol, another effect that is specific to the MPOA (Roselli and Fasasi, 1992). In the MeA and BnST there are high concentrations of aromatase but its expression is testosterone independent (Roselli et al 1991, Roselli et al 1997, Resko et al. 2000). Aromatase expression in the MPOA was decreased in aged males, but there was

no difference between young and aged castrates given testosterone replacement in the amount of increased aromatase expression, and aged animals with increased aromatase expression continued to show impairments in sexual behavior (Chambers et al. 1991). While estradiol replacement in castrates does not maintain aromatase expression, aromatase and ERalpha are colocalized in the musk shrew POA quail medial preoptic area (POM). There is no age related change in the number of cells which colocalize aromatase and ERalpha, but there is an age related decrease in single labelled aromatase cells (Dellovade 1995, Greco 1998).

6.3 The MeA and BnST in Sexual Behavior and Aging

My study on mRNA expression in response to estradiol did show most of the age-related changes in the MeA and BnST, both of which are involved in the chemosensoy system and male sexual behavior. Steroid sensitive hormones in the MeA are activated by sexual behavior and project to both the POA and BnST (Hull and Dominguez, 2006). The BnST also projects to the POA and back to the MeA (Been and Petrulis 2011, Hull and Dominguez 2007). Lesions of the MeA or BnST, in contrast to POA lesions, will not prevent copulation, but will result primarily deficits in motivated behaviors, such as partner preference and mount latency (Hull and Dominguez 2007). Both AR and ER are expressed in the MeA, and activated by either olfactory cues or copulation (Roselli et al. 1991, Yeh et al. 2009, Hull and Dominguez, 2007). Aromatase is highly expressed in both the MeA and BnST, but is not regulated by androgen receptor (Tabatdze et al. 2014, Roselli et al. 1991, Resko et al. 2000). Aromatase knockouts copulated normally, but did not show partner preference and had longer mount and intromission latencies (Bakker et al. 2002). ERalpha and ERbeta in the MeA are also both necessary for sexual behavior, instead of

just ERalpha, as is seen in the MPOA (Sano et al. 2013, Russell et al. 2012). ERbeta decreased in aged rats in the MeA, but any associated effects on sexual behavior were not investigated (Yamaguchi and Yuri, 2012). More studies are needed to examine age-related changes in estrogen receptor expression and response to hormone administration in the MeA in males, as well as the contributions of each type to sexual behavior in aged animals.

6.4 CHANGES IN AGING, SATIETY AND NON-COPULATING MALES

Age-related impairments in sexual behavior a differ from deficits seen in non-copulating (NC) or sexually sated males. Castration decreases AR expression, which is restored by testosterone replacement (Wood and newman 1993), and AR density and AR mRNA in the MPOA are both decreased during satiety (Fernandez-Guasti et al. 2010, Romano-Torres et al. 2007). Testosterone or estradiol implants in the MPOA of NC or castrated males stimulated mating, and testosterone administration increased the percentage of animals ejaculating 48 hours after satiety (Phillips-Farfan 2008, Antonio-Cabrera 2014, Russell 2012), but aged animals with hormone replacement continue to show age-related deficits (Chambers et al. 1991, Chambers and Phoenix 1984, Wu and Gore 2009). While decreased AR binding the preoptic area has was seen in a study of aged males who no longer copulated, castration and testosterone replacement increased AR expression to levels comparable with young intact males, but only restored copulation in 25% of males (Chambers 1991). Other studies showed increased AR expression in aged males showing deficits in sexual behavior, as well as replicating the finding that testosterone in these aged males continued to increase AR expression compared to castrates without restoring behavior in aged animals to levels seen in young

castrates with testosterone replacement (Wu and Gore 2009, Wu and Gore 2010). ERalpha is decreased in the MPOA of NC, but does not change during aging, and increases during sexual satiety (Wu and Gore 2009, Portillo 2006, Phillips-Farfan 2007). It is also suspected that different mechanisms are involved in sexual behavior vs. satiety, meaning age-related changes in copulatory behavior would not necessarily mirror those seen in satiety (Hull and Domingeuz 2007, Fernandez-Guasti and Rodriguez-Manzo 2003, Everitt 1990).

6.5 Steroid Hormones and Steroid Receptor Modulators in Humans

In humans selective estrogen receptor modulators (SERM) and aromatase inhibitors (AI) are used to treat cancer, hypogonadism, infertility and schizophrenia (Fentiman 2016, Ring et al 2016, Kindler et al. 2015). Treatment of hypogonadism with aromatase inhibitors or SERMs, instead of testosterone replacement, has become more common (McBride et al. 2015). Although treatment with these drugs does raise testosterone concentrations in men, there are still some concerns surrounding the possibility of unknown side effects as they are better studied in women, and what even qualifies as low testosterone in men is poorly defined (McBride et al 2015). Confusion over how to diagnose or treat testosterone deficiency or prostate cancer in men in clinical practice is evidenced by the fact that 93% of Canadian urologists are involved in the treatment of a patient with prostate cancer, only 65% said they would offer testosterone replacement therapy (TRT) to a patient with a low stage of prostate cancer, despite believing it would not increase the risk of cancer progression, and only 35% had ever actually offered TRT in their practice (Millar et al. 2016). Though it is known that low

testosterone may lead to decreased libido and sexual function, one of tamoxifen's side effects is decreasing libido in either cancer patients or hypogonadal men, despite increasing serum testosterone concentrations (McBride et al. 2015). Another use of AIs and SERMs is in male breast cancer patients. A greater proportion of male breast cancer patients have estrogen receptor positive breast cancer compared to women, and show better outcomes and reduced mortality rates using SERMs like tamoxifen instead of AIs. However, one study found that 20% of the male breast cancer patients in their study did not complete treatment when given tamoxifen due to side effects, while another found that only 17.7% of patients given a five year tamoxifen prescription completed it (Pemmaraju et al. 2012, Xu et al. 2012). A better understanding of how estradiol and estrogen receptors function in men, young and old, could lead to more effective treatment with fewer side effects.

6.4 Conclusion

Estradiol plays an important role in male sexual behavior, the regulation of the male HPG-axis, and expression of multiple hormones, receptors and neurotransmitters. Age alters how nuclei in males respond to estradiol in a region-specific manner. There is a complex circuit underlying the expression of male sexual behavior, and we need to understand not just how they interact, but which regions are more susceptible to age related changes which could lead to behavioral impairments. Changes in how males respond to steroid hormone have implications for not only male sexual behavior, but men given AIs and SERMs to treat a variety of conditions. A better understanding of estradiol

in aging males will both expand our general knowledge of steroid hormone function and could provide better targets for drug therapies in humans.

Bibliography

Adams, M. M., Oung, T., Morrison, J. H., & Gore, A. C. (2001). Length of postovariectomy interval and age, but not estrogen replacement, regulate N-methyl-D-aspartate receptor mRNA levels in the hippocampus of female rats. *Experimental Neurology*, *170*(2), 345–56. http://doi.org/10.1006/exnr.2001.7716

Aenlle, K. K., & Foster, T. C. (2010). Aging alters the expression of genes for neuroprotection and synaptic function following acute estradiol treatment. *Hippocampus*, *20*(9), 1047–60. http://doi.org/10.1002/hipo.20703

Alyea, R. A., Laurence, S. E., Kim, S. H., Katzenellenbogen, B. S., Katzenellenbogen, J. A., & Watson, C. S. (2008). The roles of membrane estrogen receptor subtypes in modulating dopamine transporters in PC-12 cells. *Journal of Neurochemistry*, *106*(4), 1525–33. http://doi.org/10.1111/j.1471-4159.2008.05491.x

Amstislavskaia, T. G., Gladkikh, D. V, Belousova, I. I., Maslova, L. N., & Kolosova, N. G. (2010). [Age-related aspects of male rats sexual behavior with different senescence rates]. *Zhurnal Vysshet Nervnoĭ Deiatelnosti Imeni I P Pavlova*, *60*(5), 552–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21260979

Antonio-Cabrera, E., & Paredes, R. G. (2014). Testosterone or oestradiol implants in the medial preoptic area induce mating in noncopulating male rats. *Journal of Neuroendocrinology*, *26*(7), 448–58. http://doi.org/10.1111/jne.12164

Arendash, G. W., & Gorski, R. a. (1983). Effects of discrete lesions of the sexually dimorphic nucleus of the preoptic area or other medial preoptic regions on the sexual behavior of male rats. *Brain Research Bulletin*, *10*(1), 147–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6824962

Arimoto, J. M., Wong, A., Rozovsky, I., Lin, S. W., Morgan, T. E., & Finch, C. E. (2013). Age increase of estrogen receptor-α (ERα) in cortical astrocytes impairs neurotrophic support in male and female rats. *Endocrinology*, *154*(6), 2101–13. http://doi.org/10.1210/en.2012-2046

Attila, M., Oksala, R., & Ågmo, A. (2009). Sexual incentive motivation in male rats requires both androgens and estrogens. *Hormones and Behavior*. http://doi.org/10.1016/j.yhbeh.2009.08.011

Bakker, J., Honda, S., Harada, N., & Balthazart, J. (2002). Sexual partner preference requires a functional aromatase (cyp19) gene in male mice. *Hormones and Behavior*, *42*(2), 158–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12367569

Balthazart, J., Baillien, M., & Ball, G. F. (2002). Interactions between aromatase and dopamine in the control of male sexual behavior in quail. *Comp. Biochem. Physiol, B*, *132*, 37–55.

Balthazart, J., & Ball, G. F. (2007). Topography in the preoptic region: differential regulation of appetitive and consummatory male sexual behaviors. *Front*.

Balthazart, J., Baillien, M., & Ball, G. F. (2002). Phosphorylation processes mediate rapid changes of brain aromatase activity ☪. *Journal of Steroid Biochemistry*, *79*(2001), 261–277.

Been, L. E., & Petrulis, A. (2011). Chemosensory and hormone information are relayed directly between the medial amygdala, posterior bed nucleus of the stria terminalis, and medial preoptic area in male Syrian hamsters. *Hormones and Behavior*, *59*(4), 536–48. http://doi.org/10.1016/j.yhbeh.2011.02.005

Benelli, A., Bertolini, A., Poggioli, R., Cavazzuti, E., Calzà, L., Giardino, L., & Arletti, R. (1995). Nitric oxide is involved in male sexual behavior of rats. *European Journal of Pharmacology*, *294*(2), 505–510. http://doi.org/10.1016/0014-2999(95)00578-1

Bergendahl, M., Aloi, J. A., Iranmanesh, A., Mulligan, T. M., & Veldhuis, J. D. (1998). Fasting suppresses pulsatile luteinizing hormone (LH) secretion and enhances orderliness of LH release in young but not older men. *The Journal of Clinical Endocrinology and Metabolism*, *83*(6), 1967–75. http://doi.org/10.1210/jcem.83.6.4856

Bhat, G., Mahesh, V. B., Aguan, K., & Brann, D. W. (1996). Evidence that brain nitric oxide synthase is the major nitric oxide synthase isoform in the hypothalamus of the adult female rat and that nitric oxide potently regulates hypothalamic cGMP levels. *Neuroendocrinology*, *64*(2), 93–102. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8857603

Bialy, M., Rydz, M., & Kaczmarek, L. (2000). Precontact 50-kHz vocalizations in male rats during acquisition of sexual experience. *Behavioral Neuroscience*, *114*(5), 983–90. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11085613

Bian, C., Zhu, H., Zhao, Y., Cai, W., & Zhang, J. (2014). Intriguing Roles of Hippocampus-Synthesized 17β-Estradiol in the Modulation of Hippocampal Synaptic Plasticity. *Journal of Molecular Neuroscience : MN*. http://doi.org/10.1007/s12031-014-0285-8

Bimonte-Nelson, H. A., Acosta, J. I., & Talboom, J. S. (2010). Neuroscientists as cartographers: mapping the crossroads of gonadal hormones, memory and age using animal models. *Molecules (Basel, Switzerland)*, *15*(9), 6050–105. http://doi.org/10.3390/molecules15096050

Bodo, C., & Rissman, E. F. (2006). New roles for estrogen receptor beta in behavior and neuroendocrinology. *Frontiers in Neuroendocrinology*, *27*(2), 217–32. http://doi.org/10.1016/j.yfrne.2006.02.004

Böhme, G. A., Bon, C., Lemaire, M., Reibaud, M., Piot, O., Stutzmann, J. M., … Blanchard, J. C. (1993). Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(19), 9191–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7692445

Bonavera, J. J., Swerdloff, R. S., Leung, A., Lue, Y. H., Baravarian, S., Superlano, L., … Wang, C. (1997). In the male brown-Norway (BN) male rat, reproductive aging is associated with decreased LH-pulse amplitude and area. *Journal of Andrology*, *18*(4), 359–65. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9283947

Bondar, G., Kuo, J., Hamid, N., & Micevych, P. (2009). Estradiol-induced estrogen receptor-alpha trafficking. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(48), 15323–30. http://doi.org/10.1523/JNEUROSCI.2107-09.2009

Böttner, M., Leonhardt, S., Wuttke, W., & Jarry, H. (2007). Changes of Expression of Genes Related to the Activity of the Gonadotrophin-Releasing Hormone Pulse Generator in Young Versus Middle-Aged Male Rats. *Journal of Neuroendocrinology*, *19*(10), 779–787. http://doi.org/10.1111/j.1365-2826.2007.01589.x

Bredt, D. S., & Snyder, S. H. (1992). Nitric oxide, a novel neuronal messenger. *Neuron*, *8*(1), 3–11. http://doi.org/10.1016/0896-6273(92)90104-L

Bronson, F. H., & Desjardins, C. (1982). Endocrine responses to sexual arousal in male mice. *Endocrinology*, *111*(4), 1286–91. http://doi.org/10.1210/endo-111-4-1286

Calcagnoli, F., de Boer, S. F., Beiderbeck, D. I., Althaus, M., Koolhaas, J. M., & Neumann, I. D. (2014). Local oxytocin expression and oxytocin receptor binding in the male rat brain is associated with aggressiveness. *Behavioural Brain Research*, *261*, 315–322. http://doi.org/10.1016/j.bbr.2013.12.050

Chambers, K. C., & Phoenix, C. H. (1984). Testosterone and the decline of sexual behavior in aging male rats. *Behavioral and Neural Biology*, *40*(1), 87–97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6732707

Chambers, K. C., & Phoenix, C. H. (1986). Testosterone is more effective than dihydrotestosterone plus estradiol in activating sexual behavior in old male rats. *Neurobiology of Aging*, *7*(2), 127–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3960265

Chambers, K. C., Thornton, J. E., & Roselli, C. E. (1991). Age-related deficits in brain androgen binding and metabolism, testosterone, and sexual behavior of male rats. *Neurobiology of Aging*, *12*(2), 123–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2052127

Charlier, T. D., Cornil, C. A., Ball, G. F., & Balthazart, J. (2010). Diversity of mechanisms involved in aromatase regulation and estrogen action in the brain. *Biochimica et Biophysica Acta*, *1800*(10), 1094–105. http://doi.org/10.1016/j.bbagen.2009.12.010

Chen, J. C., Tsai, H., Yeh, K., Tai, M., & Tsai, Y. (2007). Male sexual behavior and catecholamine levels in the medial preoptic area and arcuate nucleus in middle-aged rats. *Group*, *4*, 2–8. http://doi.org/10.1016/j.brainres.2007.10.003

Chung, S. K., McCabe, J. T., & Pfaff, D. W. (1991). Estrogen influences on oxytocin mRNA expression in preoptic and anterior hypothalamic regions studied by in situ hybridization. *The Journal of Comparative Neurology*, *307*, 281–295. http://doi.org/10.1002/cne.903070209

Clarke, H., Dhillo, W. S., & Jayasena, C. N. (2015). Comprehensive Review on Kisspeptin and Its Role in Reproductive Disorders. *Endocrinology and Metabolism (Seoul, Korea)*, *30*(2), 124–41. http://doi.org/10.3803/EnM.2015.30.2.124

Conrad, C. D., & Bimonte-Nelson, H. A. (2010). Impact of the hypothalamic-pituitary-adrenal/gonadal axes on trajectory of age-related cognitive decline. *Progress in Brain Research*, *182*, 31–76. http://doi.org/10.1016/S0079-6123(10)82002-3

Coolen, L. M., Peters, H. J., & Veening, J. G. (1997). Distribution of Fos immunoreactivity following mating versus anogenital investigation in the male rat brain. *Neuroscience*, *77*(4), 1151–61. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9130794

Cornil, C. A., & Ball, G. F. (2010). Effects of social experience on subsequent sexual performance in naïve male Japanese quail (Coturnix japonica). *Hormones and Behavior*, *57*(4–5), 515–522. http://doi.org/10.1016/j.yhbeh.2010.02.010

Cross, E., & Roselli, C. E. (1999). 17Beta-Estradiol Rapidly Facilitates Chemoinvestigation and Mounting in Castrated Male Rats. *The American Journal of Physiology*, *276*(5 Pt 2), R1346–R1350.

d'Anglemont de Tassigny, X., Campagne, C., Dehouck, B., Leroy, D., Holstein, G. R., Beauvillain, J.-C., … Prevot, V. (2007). Coupling of neuronal nitric oxide synthase to NMDA receptors via postsynaptic density-95 depends on estrogen and contributes to the central control of adult female reproduction. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(23), 6103–14. http://doi.org/10.1523/JNEUROSCI.5595-06.2007

d'Anglemont de Tassigny, X., Campagne, C., Steculorum, S., & Prevot, V. (2009). Estradiol induces physical association of neuronal nitric oxide synthase with NMDA receptor and promotes nitric oxide formation via estrogen receptor activation in primary neuronal cultures. *Journal of Neurochemistry*, *109*(1), 214–24. http://doi.org/10.1111/j.1471-4159.2009.05949.x

Dawson, V. L., Sasaki, M., & Gonzalez-Zulueta, M. (1998). Regulation of neuronal nitric oxide synthase and identification of novel nitric oxide signaling pathways. *Progress in Brain Research*, *118*, 3–11. http://doi.org/10.1016/S0079-6123(08)63196-9

Dellovade, T. L., Rissman, E. F., Thompson, N., Harada, N., & Ottinger, M. A. (1995). Co-localization of aromatase enzyme and estrogen receptor immunoreactivity in the preoptic area during reproductive aging. *Brain Research*, *674*(2), 181–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7796096

Dewing, P., Boulware, M. I., Sinchak, K., Christensen, A., Mermelstein, P. G., & Micevych, P. (2007). Membrane estrogen receptor-alpha interactions with metabotropic glutamate receptor 1a modulate female sexual receptivity in rats. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(35), 9294–300. http://doi.org/10.1523/JNEUROSCI.0592-07.2007

Dobado-Berrios, P. M., Ruíz-Navarro, A., Almadén, Y., Malagón, M. M., Garrido, J. C., Ramírez-Gutiérrez, J. L., & Gracia-Navarro, F. (1996). Heterogeneity of growth hormone (GH)-producing cells in aging male rats: ultrastructure and GH gene expression in somatotrope subpopulations. *Molecular and Cellular Endocrinology*, *118*(1–2), 181–91. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8735604

Dominguez, J. M., & Hull, E. M. (2005). Dopamine, the medial preoptic area, and male sexual behavior. *Physiol. Behav.*, *86*(3), 356–368.

Dominguez, J. M., & Hull, E. M. (2001). Stimulation of the medial amygdala enhances medial preoptic dopamine release: implications for male rat sexual behavior. *Brain Research*, *917*(2), 225–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11640908

Dominguez, J. M., Muschamp, J. W., Schmich, J. M., & Hull, E. M. (2004). Nitric oxide mediates glutamate-evoked dopamine release in the medial preoptic area. *Neuroscience*, *125*(1), 203–210.

Dominguez, J. M. (2006). Preoptic Glutamate Facilitates Male Sexual Behavior. *Journal of Neuroscience*, *26*(6), 1699–1703. http://doi.org/10.1523/JNEUROSCI.4176-05.2006

Dominguez, J. M. (2009). A role for preoptic glutamate in the regulation of male reproductive behavior. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *15*(1), 11–9. http://doi.org/10.1177/1073858408322679

Druzin, M., Malinina, E., Grimsholm, O., & Johansson, S. (2011). Mechanism of estradiol-induced block of voltage-gated K+ currents in rat medial preoptic neurons. *PloS One*, *6*(5), e20213. http://doi.org/10.1371/journal.pone.0020213

Dumais, K. M., Bredewold, R., Mayer, T. E., & Veenema, A. H. (2013). Sex differences in oxytocin receptor binding in forebrain regions: Correlations with social interest in brain region- and sex- specific ways. *Hormones and Behavior*, *64*(4), 693–701. http://doi.org/10.1016/j.yhbeh.2013.08.012

El-Emam Dief, A., Caldwell, J. D., & Jirikowski, G. F. (2013). Colocalization of P450 aromatase and oxytocin immunostaining in the rat hypothalamus. *Hormone and Metabolic Research*, *45*, 273–276. http://doi.org/10.1055/s-0032-1327680

Ervin, K. S. J., Lymer, J. M., Matta, R., Clipperton-Allen, A. E., Kavaliers, M., & Choleris, E. (2015). Estrogen involvement in social behavior in rodents: Rapid and long-term actions. *Hormones and Behavior*, *74*, 53–76. http://doi.org/10.1016/j.yhbeh.2015.05.023

Estes, K. S., & Simpkins, J. W. (1980). Age-related alterations in catecholamine concentrations in discrete preoptic area and hypothalamic regions in the male rat. *Brain Research*, *194*(2), 556–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7388632

Everitt, B. J. (1990). Sexual motivation: a neural and behavioural analysis of the mechanisms underlying appetitive and copulatory responses of male rats. *Neuroscience and Biobehavioral Reviews*, *14*(2), 217–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2190121

Feil, R., & Kleppisch, T. (2008). NO/cGMP-dependent modulation of synaptic transmission. *Handbook of Experimental Pharmacology*, (184), 529–60. http://doi.org/10.1007/978-3-540-74805-2_16

Fentiman, I. S. (2016). Male breast cancer is not congruent with the female disease. *Critical Reviews in Oncology/hematology*, *101*, 119–24. http://doi.org/10.1016/j.critrevonc.2016.02.017

Fernández-Guasti, A., Arteaga-López, P., & Antonio-Cabrera, E. (2010). Copulation modifies AR and ERα mRNA expression in the male rat brain. *Physiology & Behavior*, *101*(5), 738–45. http://doi.org/10.1016/j.physbeh.2010.08.015

Fernández-Guasti, A., & Rodríguez-Manzo, G. (2003). Pharmacological and physiological aspects of sexual exhaustion in male rats. *Scandinavian Journal of Psychology*, *44*(3), 257–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12914589

Foster, T. C. (2012). Role of estrogen receptor alpha and beta expression and signaling on cognitive function during aging. *Hippocampus*, *22*(4), 656–69. http://doi.org/10.1002/hipo.20935

Foster, T. C., Defazio, R. A., & Bizon, J. L. (2012). Characterizing cognitive aging of spatial and contextual memory in animal models. *Frontiers in Aging Neuroscience*, *4*, 12. http://doi.org/10.3389/fnagi.2012.00012

Foy, M. R., Baudry, M., Akopian, G. K., & Thompson, R. F. (2010). Regulation of hippocampal synaptic plasticity by estrogen and progesterone. *Vitamins and Hormones*, *82*, 219–39. http://doi.org/10.1016/S0083-6729(10)82012-6

Franklin, T. B., & Perrot-Sinal, T. S. (2006). Sex and ovarian steroids modulate brain-derived neurotrophic factor (BDNF) protein levels in rat hippocampus under stressful and non-stressful conditions. *Psychoneuroendocrinology*, *31*(1), 38–48. http://doi.org/10.1016/j.psyneuen.2005.05.008

Fugger, H. N., Kumar, A., Lubahn, D. B., Korach, K. S., & Foster, T. C. (2001). Examination of estradiol effects on the rapid estradiol mediated increase in hippocampal synaptic transmission in estrogen receptor alpha knockout mice.

Neuroscience Letters, *309*(3), 207–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11514077

Fujita, S., Chiba, M., Ohta, M., Kitani, K., & Suzuki, T. (1990). Alteration of plasma sex hormone levels associated with old age and its effect on hepatic drug metabolism in rats. *The Journal of Pharmacology and Experimental Therapeutics*, *253*(1), 369–74. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2329519

Garthwaite, J., & Boulton, C. L. (1995). Nitric Oxide Signaling in the Central Nervous System. *Annual Review of Physiology*, *57*(1), 683–706. http://doi.org/10.1146/annurev.ph.57.030195.003343

Garthwaite, J., Charles, S. L., & Chess-Williams, R. (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature*, *336*(6197), 385–388. http://doi.org/10.1038/336385a0

Gates, M. A., Mekary, R. A., Chiu, G. R., Ding, E. L., Wittert, G. A., & Araujo, A. B. (2013). Sex steroid hormone levels and body composition in men. *The Journal of Clinical Endocrinology and Metabolism*, *98*(6), 2442–50. http://doi.org/10.1210/jc.2012-2582

Gautier, A., Bonnet, F., Dubois, S., Massart, C., Grosheny, C., Bachelot, A., … Ducluzeau, P.-H. (2013). Associations between visceral adipose tissue, inflammation and sex steroid concentrations in men. *Clinical Endocrinology*, *78*(3), 373–8. http://doi.org/10.1111/j.1365-2265.2012.04401.x

Gil, M., Bhatt, R., Picotte, K. B., & Hull, E. M. (2011). Oxytocin in the medial preoptic area facilitates male sexual behavior in the rat. *Hormones and Behavior*, *59*(4), 435–443. http://doi.org/10.1016/j.yhbeh.2010.12.012

Gingerich, S., & Krukoff, T. L. (2005). Estrogen modulates endothelial and neuronal nitric oxide synthase expression via an estrogen receptor beta-dependent mechanism in hypothalamic slice cultures. *Endocrinology*, *146*(7), 2933–41. http://doi.org/10.1210/en.2004-1375

Gingerich, S., & Krukoff, T. L. (2006). Estrogen in the paraventricular nucleus attenuates L-glutamate-induced increases in mean arterial pressure through estrogen receptor beta and NO. *Hypertension (Dallas, Tex. : 1979)*, *48*(6), 1130–6. http://doi.org/10.1161/01.HYP.0000248754.67128.ff

Gooren, L. (1989). Androgens and estrogens in their negative feedback action in the hypothalamo-pituitary-testis axis: site of action and evidence of their interaction.

Journal of Steroid Biochemistry, *33*(4B), 757–61. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2689784

Gore, A. C. (1998). Diurnal rhythmicity of gonadotropin-releasing hormone gene expression in the rat. *Neuroendocrinology*, *68*(4), 257–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9772340

Goya, R. G., Lu, J. K. H., & Meites, J. (1990). Gonadal function in aging rats and its relation to pituitary and mammary pathology. *Mechanisms of Ageing and Development*, *56*(1), 77–88. http://doi.org/10.1016/0047-6374(90)90116-W

Gréco, B., Edwards, D. A., Michael, R. P., & Clancy, A. N. (1998). Androgen receptors and estrogen receptors are colocalized in male rat hypothalamic and limbic neurons that express Fos immunoreactivity induced by mating. *Neuroendocrinology*, *67*(1), 18–28. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9485165

Greenblatt, R. B., Oettinger, M., & Bohler, C. S. (1976). Estrogen-androgen levels in aging men and women: therapeutic considerations. *Journal of the American Geriatrics Society*, *24*(4), 173–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1254880

Gruenewald, D. A., Hess, D. L., Wilkinson, C. W., & Matsumoto, A. M. (1992). Excessive testicular progesterone secretion in aged male Fischer 344 rats: a potential cause of age-related gonadotropin suppression and confounding variable in aging studies. *Journal of Gerontology*, *47*(5), B164-70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1512426

Gruenewald, D. A., Naai, M. A., Hess, D. L., & Matsumoto, A. M. (1994). The Brown Norway rat as a model of male reproductive aging: evidence for both primary and secondary testicular failure. *Journal of Gerontology*, *49*(2), B42-50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8126345

Gruenewald, D. A., Naai, M. A., Marck, B. T., & Matsumoto, A. M. (2000). Age-related decrease in hypothalamic gonadotropin-releasing hormone (GnRH) gene expression, but not pituitary responsiveness to GnRH, in the male Brown Norway rat. *Journal of Andrology*, *21*(1), 72–84. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10670522

Haji, M., Kato, K. I., Nawata, H., & Ibayashi, H. (1981). Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat. *Brain Research*, *204*(2), 373–86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6780133

Hamson, D. K., Csupity, A. S., Ali, F. M., & Watson, N. V. (2009). Partner preference and mount latency are masculinized in androgen insensitive rats. *Physiology & Behavior*, *98*(1), 25–30. http://doi.org/10.1016/j.physbeh.2009.04.008

Harburger, L. L., Saadi, a, & Frick, K. M. (2009). Dose-dependent effects of post-training estradiol plus progesterone treatment on object memory consolidation and hippocampal extracellular signal-regulated kinase activation in young ovariectomized mice. *Neuroscience*, *160*(1), 6–12. http://doi.org/10.1016/j.neuroscience.2009.02.024

Hawkins, R. D., Son, H., & Arancio, O. (1998). Nitric oxide as a retrograde messenger during long-term potentiation in hippocampus. *Progress in Brain Research*, *118*, 155–172. http://doi.org/10.1016/S0079-6123(08)63206-9

Hayes, F. J., Seminara, S. B., Decruz, S., Boepple, P. A., & Crowley, W. F. (2000). Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback. *The Journal of Clinical Endocrinology and Metabolism*, *85*(9), 3027–35. http://doi.org/10.1210/jcem.85.9.6795

Heaton, J. P. (2003). Hormone treatments and preventive strategies in the aging male: whom and when to treat? *Reviews in Urology*, *5 Suppl 1*(Suppl 1), S16-21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16985938

Herath, C. B., Watanabe, G., Wanzhu, J., Noguchi, J., Akiyama, K., Kuramoto, K., … Taya, K. (2001). Elevated levels of inhibin-A and immunoreactive inhibin in aged male Wistar rats with testicular Leydig cell tumor. *Journal of Andrology*, *22*(5), 838–46. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11545298

Herrera-Pérez, J. J., Martínez-Mota, L., & Fernández-Guasti, A. (2008). Aging increases the susceptibility to develop anhedonia in male rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *32*(8), 1798–1803. http://doi.org/10.1016/j.pnpbp.2008.07.020

Hogervorst, E., Bandelow, S., & Moffat, S. D. (2005). Increasing testosterone levels and effects on cognitive functions in elderly men and women: a review. *Current Drug Targets. CNS and Neurological Disorders*, *4*(5), 531–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16266286

Hrabovszky, E., Kalló, I., Szlávik, N., Keller, E., Merchenthaler, I., & Liposits, Z. (2007). Gonadotropin-releasing hormone neurons express estrogen receptor-beta. *The Journal of Clinical Endocrinology and Metabolism*, *92*(7), 2827–30. http://doi.org/10.1210/jc.2006-2819

Hrabovszky, E., Steinhauser, A., Barabás, K., Shughrue, P. J., Petersen, S. L., Merchenthaler, I., & Liposits, Z. (2001). Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology*, *142*(7), 3261–4. http://doi.org/10.1210/endo.142.7.8176

Hrabovszky, E., Kalló, I., Steinhauser, A., Merchenthaler, I., Coen, C. W., Petersen, S. L., & Liposits, Z. (2004). Estrogen receptor-beta in oxytocin and vasopressin neurons of the rat and human hypothalamus: Immunocytochemical and in situ hybridization studies. *The Journal of Comparative Neurology*, *473*(3), 315–33. http://doi.org/10.1002/cne.20127

Hu, L., Gustofson, R. L., Feng, H., Leung, P. K., Mores, N., Krsmanovic, L. Z., & Catt, K. J. (2008). Converse regulatory functions of estrogen receptor-alpha and -beta subtypes expressed in hypothalamic gonadotropin-releasing hormone neurons. *Molecular Endocrinology (Baltimore, Md.)*, *22*(10), 2250–9. http://doi.org/10.1210/me.2008-0192

Hull, E. M., Du, J., Lorrain, D. S., & Matuszewich, L. (1995). Extracellular dopamine in the medial preoptic area: implications for sexual motivation and hormonal control of copulation. *J. Neurosci.*, *15*, 7465–7471.

Hull, E. M., Du, J., Lorrain, D. S., & Matuszewich, L. (1997). Testosterone, preoptic dopamine, and copulation in male rats. *Brain Res. Bull.*, *44*(4), 327–333.

Hull, E. M., Lumley, L. A., Matuszewich, L., Dominguez, J., Moses, J., & Lorrain, D. S. (1994). The Roles of Nitric Oxide in Sexual Function of Male Rats, *33*, 1499–504.

Hull, E. M., Muschamp, J. W., & Sato, S. (2004). Dopamine and serotonin: influences on male sexual behavior. *Physiol. Behav.*, *83*, 291–307.

Hull, E. M., & Dominguez, J. M. (2006). Getting his act together : Roles of glutamate , nitric oxide , and dopamine in the medial preoptic area, *26*. http://doi.org/10.1016/j.brainres.2006.08.031

Hull, E. M., & Dominguez, J. M. (2007). Sexual behavior in male rodents. *Hormones and Behavior*, *52*, 45–55. http://doi.org/10.1016/j.yhbeh.2007.03.030

Iranmanesh, A., Mulligan, T., & Veldhuis, J. D. (2010). Age in men does not determine gonadotropin-releasing hormone's dose-dependent stimulation of luteinizing hormone secretion under an exogenous testosterone clamp. *The Journal of Clinical Endocrinology and Metabolism*, *95*(6), 2877–84. http://doi.org/10.1210/jc.2009-2581

Izumo, N., Ishibashi, Y., Ohba, M., Morikawa, T., & Manabe, T. (2012). Decreased voluntary activity and amygdala levels of serotonin and dopamine in ovariectomized rats. *Behavioural Brain Research*, *227*(1), 1–6. http://doi.org/10.1016/j.bbr.2011.10.031

Janowsky, J. S. (2006). The role of androgens in cognition and brain aging in men. *Neuroscience*, *138*(3), 1015–20. http://doi.org/10.1016/j.neuroscience.2005.09.007

Jasuja, G. K., Travison, T. G., Davda, M., Murabito, J. M., Basaria, S., Zhang, A., … Bhasin, S. (2013). Age trends in estradiol and estrone levels measured using liquid chromatography tandem mass spectrometry in community-dwelling men of the Framingham Heart Study. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *68*(6), 733–40. http://doi.org/10.1093/gerona/gls216

Kamel, F., & Frankel, A. (1978). Hormone Release during Mating in the Male Rat: Time Course, Relation to Sexual Behavior, and Interaction with Handling Procedures*. *Endocrinology*, *103*(6), 2172–2179. http://doi.org/10.1210/endo-103-6-2172

Katoh-Semba, R., Semba, R., Takeuchi, I. K., & Kato, K. (1998). Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescence-accelerated mice: a comparison to those of nerve growth factor and neurotrophin-3. *Neuroscience Research*, *31*(3), 227–34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9809668

Keenan, D. M., Clarke, I. J., & Veldhuis, J. D. (2011). Noninvasive analytical estimation of endogenous GnRH drive: analysis using graded competitive GnRH-receptor antagonism and a calibrating pulse of exogenous GnRH. *Endocrinology*, *152*(12), 4882–93. http://doi.org/10.1210/en.2011-1461

Kendrick, K. M., Guevara-Guzman, R., Zorrilla, J., Hinton, M. R., Broad, K. D., Mimmack, M., & Ohkura, S. (1997). Formation of olfactory memories mediated by nitric oxide. *Nature*, *388*(6643), 670–674. http://doi.org/10.1038/41765

Khosla, S., Melton, L. J., Atkinson, E. J., & O'Fallon, W. M. (2001). Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *The Journal of Clinical Endocrinology and Metabolism*, *86*(8), 3555–61. http://doi.org/10.1210/jcem.86.8.7736

Kindler, J., Weickert, C. S., Skilleter, A. J., Catts, S. V, Lenroot, R., & Weickert, T. W. (2015). Selective Estrogen Receptor Modulation Increases Hippocampal Activity during Probabilistic Association Learning in Schizophrenia. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *40*(10), 2388–97. http://doi.org/10.1038/npp.2015.88

Kudwa, A. E., Gustafsson, J.-A., & Rissman, E. F. (2004). Estrogen receptor beta modulates estradiol induction of progestin receptor immunoreactivity in male, but not in female, mouse medial preoptic area. *Endocrinology*, *145*(10), 4500–6. http://doi.org/10.1210/en.2003-1708

Lagoda, G., Muschamp, J. W., Vigdorchik, A., & Hull, E. M. (2004). A Nitric Oxide Synthesis Inhibitor in the Medial Preoptic Area Inhibits Copulation and Stimulus Sensitization in Male Rats. *Behavioral Neuroscience*, *118*(6), 1317–1323. http://doi.org/10.1037/0735-7044.118.6.1317

Lakshman, K. M., Kaplan, B., Travison, T. G., Basaria, S., Knapp, P. E., Singh, A. B., … Bhasin, S. (2010). The effects of injected testosterone dose and age on the conversion of testosterone to estradiol and dihydrotestosterone in young and older men. *The Journal of Clinical Endocrinology and Metabolism*, *95*(8), 3955–64. http://doi.org/10.1210/jc.2010-0102

Larsson, K. (1959). Experience and Maturation in the Development of Sexual Behaviour in Male Puberty Rat. *Behaviour*, *14*(1), 101–107. http://doi.org/10.1163/156853959X00027

Lebow, M. A., & Chen, A. (2016). Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Molecular Psychiatry*, *21*(4), 450–463. http://doi.org/10.1038/mp.2016.1

Leifke, E., Gorenoi, V., Wichers, C., Von Zur Mühlen, A., Von Büren, E., & Brabant, G. (2000). Age-related changes of serum sex hormones, insulin-like growth factor-1 and sex-hormone binding globulin levels in men: cross-sectional data from a healthy male cohort. *Clinical Endocrinology*, *53*(6), 689–95. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11155090

Lisk, R. D., & Heimann, J. (1980). The effects of sexual experience and frequency of testing on retention of copulatory behavior following castration in the male hamster. *Behavioral and Neural Biology*, *28*(2), 156–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7362582

Liu, P. Y., Takahashi, P. Y., Roebuck, P. D., Bailey, J. N., Keenan, D. M., & Veldhuis, J. D. (2009). Testosterone's short-term positive effect on luteinizing-hormone secretory-burst mass and its negative effect on secretory-burst frequency are attenuated in middle-aged men. *The Journal of Clinical Endocrinology and Metabolism*, *94*(10), 3978–86. http://doi.org/10.1210/jc.2009-0135

Liu, P. Y., Takahashi, P. Y., Roebuck, P. D., Iranmanesh, A., & Veldhuis, J. D. (2005). Age-specific changes in the regulation of LH-dependent testosterone secretion:

assessing responsiveness to varying endogenous gonadotropin output in normal men. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *289*(3), R721-8. http://doi.org/10.1152/ajpregu.00138.2005

Liu, Y. C., Salamone, J. D., & Sachs, B. D. (1997). Lesions in medial preoptic area and bed nucleus of stria terminalis: differential effects on copulatory behavior and noncontact erection in male rats. *J. Neurosci.*, *17*, 5245–5253.

Lommatzsch, M., Zingler, D., Schuhbaeck, K., Schloetcke, K., Zingler, C., Schuff-Werner, P., & Virchow, J. C. (2005). The impact of age, weight and gender on BDNF levels in human platelets and plasma. *Neurobiology of Aging*, *26*(1), 115–23. http://doi.org/10.1016/j.neurobiolaging.2004.03.002

Lorrain, D. S., Matuszewich, L., Howard, R. V, Du, J., & Hull, E. M. (1996). Nitric oxide promotes medial preoptic dopamine release during male rat copulation. *Neuroreport*, *8*(1), 31–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9051747

Luine, V. N., Beck, K. D., Bowman, R. E., Frankfurt, M., & Maclusky, N. J. (2007). Chronic stress and neural function: accounting for sex and age. *Journal of Neuroendocrinology*, *19*(10), 743–51. http://doi.org/10.1111/j.1365-2826.2007.01594.x

Lumley, L. A., & Hull, E. M. (1999). Effects of a D antagonist and of sexual experience on copulation-induced Fos-like immunoreactivity in the medial preoptic nucleus. *Brain Research*, *829*, 55–68.

Madeira, M. D., Andrade, J. P., & Paula-Barbosa, M. M. (2000). Hypertrophy of the ageing rat medial preoptic nucleus. *Journal of Neurocytology*, *29*(3), 173–97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11428048

Malsbury, C. W. (1971). Facilitation of male rat copulatory behavior by electrical stimulation of the medial preoptic area. *Physiology & Behavior*, *7*(6), 797–805. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5134017

Mao, L., & Wang, J. Q. (2002). Glutamate cascade to cAMP response element-binding protein phosphorylation in cultured striatal neurons through calcium-coupled group I metabotropic glutamate receptors. *Molecular Pharmacology*, *62*(3), 473–84. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12181423

Maras, P. M., & Petrulis, A. (2010). Anatomical connections between the anterior and posterodorsal sub-regions of the medial amygdala: integration of odor and hormone

signals. *Neuroscience*, *170*(2), 610–622. http://doi.org/10.1016/j.neuroscience.2010.06.075

Maras, P. M., & Petrulis, A. (2006). Chemosensory and steroid-responsive regions of the medial amygdala regulate distinct aspects of opposite-sex odor preference in male Syrian hamsters. *The European Journal of Neuroscience*, *24*(12), 3541–52. http://doi.org/10.1111/j.1460-9568.2006.05216.x

Maruniak, J. A., & Bronson, F. H. (1976). Gonadotropic responses of male mice to female urine. *Endocrinology*, *99*(4), 963–9. http://doi.org/10.1210/endo-99-4-963

Matthews, J., & Gustafsson, J.-A. (2003). Estrogen signaling: a subtle balance between ER alpha and ER beta. *Molecular Interventions*, *3*(5), 281–92. http://doi.org/10.1124/mi.3.5.281

McBride, J. A., Carson, C. C., & Coward, R. M. (2015). Diagnosis and management of testosterone deficiency. *Asian Journal of Andrology*, *17*(2), 177–86. http://doi.org/10.4103/1008-682X.143317

McGinnis, M. Y., & Dreifuss, R. M. (1989). Evidence for a role of testosterone-androgen receptor interactions in mediating masculine sexual behavior in male rats. *Endocrinology*, *124*(2), 618–26. http://doi.org/10.1210/endo-124-2-618

Micevych, P., Kuo, J., & Christensen, A. (2009). Physiology of membrane oestrogen receptor signalling in reproduction. *Journal of Neuroendocrinology*, *21*(4), 249–56. http://doi.org/10.1111/j.1365-2826.2009.01833.x

Millar, A. C., Elterman, D. S., Goldenberg, L., Van Asseldonk, B., Curtis, A., & Jarvi, K. (n.d.). A survey of Canadian urologists' opinions and prescribing patterns of testosterone replacement therapy in men on active surveillance for low-risk prostate cancer. *Canadian Urological Association Journal = Journal de l'Association Des Urologues Du Canada*, *10*(5–6), 181–184. http://doi.org/10.5489/cuaj.3608

Miranda, P., Williams, C. L., & Einstein, G. (1999). Granule cells in aging rats are sexually dimorphic in their response to estradiol. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *19*(9), 3316–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10212291

Mitra, S. W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H. A., Hayashi, S., … Alves, S. E. (2003). Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. *Endocrinology*, *144*(5), 2055–67. http://doi.org/10.1210/en.2002-221069

Morissette, M., Le Saux, M., D'Astous, M., Jourdain, S., Al Sweidi, S., Morin, N., … Di Paolo, T. (2008). Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. *The Journal of Steroid Biochemistry and Molecular Biology*, *108*(3–5), 327–38. http://doi.org/10.1016/j.jsbmb.2007.09.011

Morrell, J. I., Schwanzel-Fukuda, M., Fahrbach, S. E., & Pfaff, D. W. (1984). Axonal projections and peptide content of steroid hormone concentrating neurons. *Peptides*, *5 Suppl 1*, 227–39. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6384952

Mukai, H., Kimoto, T., Hojo, Y., Kawato, S., Murakami, G., Higo, S., … Ogiue-Ikeda, M. (2010). Modulation of synaptic plasticity by brain estrogen in the hippocampus. *Biochimica et Biophysica Acta*, *1800*(10), 1030–44. http://doi.org/10.1016/j.bbagen.2009.11.002

Mulligan, T., Iranmanesh, A., Kerzner, R., Demers, L. W., & Veldhuis, J. D. (1999). Two-week pulsatile gonadotropin releasing hormone infusion unmasks dual (hypothalamic and Leydig cell) defects in the healthy aging male gonadotropic axis. *European Journal of Endocrinology / European Federation of Endocrine Societies*, *141*(3), 257–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10474123

Naugle, M. M., Nguyen, L. T., Merceron, T. K., Filardo, E., Janssen, W. G. M., Morrison, J. H., … Gore, A. C. (2014). G-protein coupled estrogen receptor, estrogen receptor α, and progesterone receptor immunohistochemistry in the hypothalamus of aging female rhesus macaques given long-term estradiol treatment. *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology*, *321*(7), 399–414. http://doi.org/10.1002/jez.1871

Navarro, A., Del Valle, E., Ordóñez, C., Martínez, E., Pérez, C., Alonso, A., … Tolivia, J. (2013). Aging and substitutive hormonal therapy influence in regional and subcellular distribution of ERα in female rat brain. *Age (Dordrecht, Netherlands)*, *35*(3), 821–37. http://doi.org/10.1007/s11357-012-9415-9

Nilsson, S., & Gustafsson, J. A. (2000). Estrogen receptor transcription and transactivation: Basic aspects of estrogen action. *Breast Cancer Research : BCR*, *2*(5), 360–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11250729

Nutsch, V. L., Will, R. G., Hattori, T., Tobiansky, D. J., & Dominguez, J. M. (2014). Sexual experience influences mating-induced activity in nitric oxide synthase-containing neurons in the medial preoptic area. *Neuroscience Letters*, *579*, 92–96. http://doi.org/10.1016/j.neulet.2014.07.021

Nyby, J. G. (2008). Reflexive testosterone release: a model system for studying the nongenomic effects of testosterone upon male behavior. *Frontiers in Neuroendocrinology*, *29*(2), 199–210. http://doi.org/10.1016/j.yfrne.2007.09.001

O'Connell, L. A., & Hofmann, H. A. (2012). Evolution of a vertebrate social decision-making network. *Science (New York, N.Y.)*, *336*(6085), 1154–7. http://doi.org/10.1126/science.1218889

Ooishi, Y., Kawato, S., Hojo, Y., Hatanaka, Y., Higo, S., Murakami, G., … Mukai, H. (2012). Modulation of synaptic plasticity in the hippocampus by hippocampus-derived estrogen and androgen. *The Journal of Steroid Biochemistry and Molecular Biology*, *131*(1–2), 37–51. http://doi.org/10.1016/j.jsbmb.2011.10.004

Orikasa, C., Kondo, Y., Hayashi, S., McEwen, B. S., & Sakuma, Y. (2002). Sexually dimorphic expression of estrogen receptor beta in the anteroventral periventricular nucleus of the rat preoptic area: implication in luteinizing hormone surge. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(5), 3306–11. http://doi.org/10.1073/pnas.052707299

Ota, K. T., Monsey, M. S., Wu, M. S., & Schafe, G. E. (2010). Synaptic Plasticity and NO-cGMP-PKG Signaling Regulate Pre- and Postsynaptic Alterations at Rat Lateral Amygdala Synapses Following Fear Conditioning. *PLoS ONE*, *5*(6), e11236. http://doi.org/10.1371/journal.pone.0011236

Ottinger, M. A. (1995). Co-localization of aromatase enzyme and estrogen receptor immunoreactivity in the preoptic area during reproductive aging b. *Young*, *674*, 181–187. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7796096

Pardo-Bellver, C., Cádiz-Moretti, B., Novejarque, A., Martínez-García, F., & Lanuza, E. (2012). Differential efferent projections of the anterior, posteroventral, and posterodorsal subdivisions of the medial amygdala in mice. *Frontiers in Neuroanatomy*, *6*, 33. http://doi.org/10.3389/fnana.2012.00033

Park, J. H., Paul, M. J., Butler, M. P., Villa, P., Burke, M., Kim, D. P., … Zucker, I. (2007). Short duration testosterone infusions maintain male sex behavior in Syrian hamsters. *Hormones and Behavior*, *52*(2), 169–76. http://doi.org/10.1016/j.yhbeh.2007.03.008

Pemmaraju, N., Munsell, M. F., Hortobagyi, G. N., & Giordano, S. H. (2012). Retrospective review of male breast cancer patients: analysis of tamoxifen-related side-effects. *Annals of Oncology : Official Journal of the European Society for Medical Oncology*, *23*(6), 1471–4. http://doi.org/10.1093/annonc/mdr459

Perea-Rodriguez, J. P., Takahashi, E. Y., Amador, T. M., Hao, R. C., Saltzman, W., & Trainor, B. C. (2015). Effects of reproductive experience on central expression of progesterone, oestrogen α, oxytocin and vasopressin receptor mRNA in male California mice (Peromyscus californicus). *Journal of Neuroendocrinology*, *27*(4), 245–52. http://doi.org/10.1111/jne.12264

Pérez, S. E., Chen, E.-Y., & Mufson, E. J. (2003). Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain. *Brain Research. Developmental Brain Research*, *145*(1), 117–39. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14519499

Peterson, C. M. (n.d.). Estrogen and progesterone receptors: an overview from the year 2000. *Journal of the Society for Gynecologic Investigation*, *7*(1 Suppl), S3-7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10732320

Pfaus, J. G., & Heeb, M. M. (n.d.). Implications of Immediate-Early Gene Induction in the Brain Following Sexual Stimulation of Female and Male Rodents.

Phillips-Farfán, B. V, Lemus, A. E., & Fernández-Guasti, A. (2007). Increased estrogen receptor alpha immunoreactivity in the forebrain of sexually satiated rats. *Hormones and Behavior*, *51*(3), 328–34. http://doi.org/10.1016/j.yhbeh.2006.12.001

Phillips-farfán, B. V, Romano-torres, M., & Fernández-guasti, A. (2008). Anabolic androgens restore mating after sexual satiety in male rats. *Training*, *89*, 241–246. http://doi.org/10.1016/j.pbb.2007.12.016

Pietranera, L., Bellini, M. J., Arévalo, M. A., Goya, R., Brocca, M. E., Garcia-Segura, L. M., & De Nicola, A. F. (2011). Increased aromatase expression in the hippocampus of spontaneously hypertensive rats: effects of estradiol administration. *Neuroscience*, *174*, 151–9. http://doi.org/10.1016/j.neuroscience.2010.11.044

Pogun, S., Baumann, M. H., & Kuhar, M. J. (1994). Nitric oxide inhibits [3H]dopamine uptake. *Brain Research*, *641*, 83–91.

Portillo, W., Díaz, N. F., Cabrera, E. A., Fernández-Guasti, A., & Paredes, R. G. (2006). Comparative analysis of immunoreactive cells for androgen receptors and oestrogen receptor alpha in copulating and non-copulating male rats. *Journal of Neuroendocrinology*, *18*(3), 168–76. http://doi.org/10.1111/j.1365-2826.2005.01401.x

Prange-Kiel, J., & Rune, G. M. (2006). Direct and indirect effects of estrogen on rat hippocampus. *Neuroscience*, *138*(3), 765–72. http://doi.org/10.1016/j.neuroscience.2005.05.061

Putnam, S. K., Sato, S., Riolo, J. V, & Hull, E. M. (2005). Effects of testosterone metabolites on copulation, medial preoptic dopamine, and NOS-immunoreactivity in castrated male rats. *Horm. Behav*, *47*, 513–522.

Putnam, S. K., Sato, S., & Hull, E. M. (2003). Effects of testosterone metabolites on copulation and medial preoptic dopamine release in castrated male rats. *Hormones and Behavior*, *44*(5), 419–426. http://doi.org/10.1016/j.yhbeh.2003.06.006

Raber, J. (2008, May). AR, apoE, and cognitive function. *Hormones and Behavior*. http://doi.org/10.1016/j.yhbeh.2008.02.012

Rao, Y. S., Mott, N. N., Wang, Y., Chung, W. C. J., & Pak, T. R. (2013). MicroRNAs in the aging female brain: a putative mechanism for age-specific estrogen effects. *Endocrinology*, *154*(8), 2795–806. http://doi.org/10.1210/en.2013-1230

Raven, G., de Jong, F. H., Kaufman, J.-M., & de Ronde, W. (2006). In men, peripheral estradiol levels directly reflect the action of estrogens at the hypothalamo-pituitary level to inhibit gonadotropin secretion. *The Journal of Clinical Endocrinology and Metabolism*, *91*(9), 3324–8. http://doi.org/10.1210/jc.2006-0462

Raven, G., Jong, F. H. de, Kaufman, J.-M., & Ronde, W. de. (2011). In Men, Peripheral Estradiol Levels Directly Reflect the Action of Estrogens at the Hypothalamo-Pituitary Level to Inhibit Gonadotropin Secretion. *http://dx.doi.org/10.1210/jc.2006-0462*.

Ren-Patterson, R. F., Cochran, L. W., Holmes, A., Lesch, K.-P., Lu, B., & Murphy, D. L. (2006). Gender-dependent modulation of brain monoamines and anxiety-like behaviors in mice with genetic serotonin transporter and BDNF deficiencies. *Cellular and Molecular Neurobiology*, *26*(4–6), 755–80. http://doi.org/10.1007/s10571-006-9048-6

Resko, J. A., Pereyra-Martinez, A. C., Stadelman, H. L., & Roselli, C. E. (2000). Region-specific regulation of cytochrome P450 aromatase messenger ribonucleic acid by androgen in brains of male rhesus monkeys. *Biology of Reproduction*, *62*(6), 1818–22. http://doi.org/10.1095/BIOLREPROD62.6.1818

Ring, J. D., Lwin, A. A., & Köhler, T. S. (2016). Current medical management of endocrine-related male infertility. *Asian Journal of Andrology*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/27072045

Rodríguez-Manzo, G., Pellicer, F., Larsson, K., & Fernández-Guasti, A. (2000). Stimulation of the medical preoptic area facilitates sexual behavior but does not

reverse sexual satiation. *Behavioral Neuroscience*, *114*(3), 553–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10883805

Romano-Torres, M., Phillips-Farfán, B. V, Chavira, R., Rodríguez-Manzo, G., & Fernández-Guasti, A. (2007). Relationship between sexual satiety and brain androgen receptors. *Neuroendocrinology*, *85*(1), 16–26. http://doi.org/10.1159/000099250

Rosario, E. R., Carroll, J., & Pike, C. J. (2010). Testosterone regulation of Alzheimer-like neuropathology in male 3xTg-AD mice involves both estrogen and androgen pathways. *Brain Research*, *1359*, 281–90. http://doi.org/10.1016/j.brainres.2010.08.068

Roselli, C. E. (1991). Sex differences in androgen receptors and aromatase activity in microdissected regions of the rat brain. *Endocrinology*, *128*(3), 1310–6. http://doi.org/10.1210/endo-128-3-1310

Roselli, C. E. (1991). Synergistic induction of aromatase activity in the rat brain by estradiol and 5 alpha-dihydrotestosterone. *Neuroendocrinology*, *53*(1), 79–84. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2046863

Roselli, C. E., Abdelgadir, S. E., & Resko, J. A. (1997). Regulation of aromatase gene expression in the adult rat brain. *Brain Research Bulletin*, *44*(4), 351–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9370199

Roselli, C. E., Cross, E., Poonyagariyagorn, H. K., & Stadelman, H. L. (2003). Role of aromatization in anticipatory and consummatory aspects of sexual behavior in male rats. *Hormones and Behavior*, *44*(2), 146–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13129487

Roselli, C. E., & Fasasi, T. A. (1992). Estradiol increases the duration of nuclear androgen receptor occupation in the preoptic area of the male rat treated with dihydrotestosterone. *The Journal of Steroid Biochemistry and Molecular Biology*, *42*(2), 161–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1567782

Roselli, C. E., & Resko, J. A. (1997). Sex differences in androgen-regulated expression of cytochrome P450 aromatase in the rat brain. *The Journal of Steroid Biochemistry and Molecular Biology*, *61*(3–6), 365–74. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9365212

Roselli, C. E., Thornton, J. E., & Chambers, K. C. (1993). Age-related deficits in brain estrogen receptors and sexual behavior of male rats. *Behavioral Neuroscience*, *107*(1), 202–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8447953

Russell, N. V, Ogaga-Mgbonyebi, E. V, Habteab, B., Dunigan, A. I., Tesfay, M. A., & Clancy, A. N. (2012). Sexual responses of the male rat medial preoptic area and medial amygdala to estrogen II: site specific effects of selective estrogenic drugs. *Hormones and Behavior*, *62*(1), 58–66. http://doi.org/10.1016/j.yhbeh.2012.04.017

Sano, K., Nakata, M., Musatov, S., Morishita, M., Sakamoto, T., Tsukahara, S., & Ogawa, S. (2016). Pubertal activation of estrogen receptor α in the medial amygdala is essential for the full expression of male social behavior in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(27), 7632–7. http://doi.org/10.1073/pnas.1524907113

Sano, K., Tsuda, M. C., Musatov, S., Sakamoto, T., & Ogawa, S. (2013). Differential effects of site-specific knockdown of estrogen receptor α in the medial amygdala, medial pre-optic area, and ventromedial nucleus of the hypothalamus on sexual and aggressive behavior of male mice. *The European Journal of Neuroscience*, *37*(8), 1308–19. http://doi.org/10.1111/ejn.12131

Schuman, E. M., & Madison, D. V. (1994). Nitric Oxide and Synaptic Function. *Annual Review of Neuroscience*, *17*(1), 153–183. http://doi.org/10.1146/annurev.ne.17.030194.001101

Scott, C. J., Tilbrook, A. J., Simmons, D. M., Rawson, J. A., Chu, S., Fuller, P. J., … Clarke, I. J. (2000). The distribution of cells containing estrogen receptor-alpha (ERalpha) and ERbeta messenger ribonucleic acid in the preoptic area and hypothalamus of the sheep: comparison of males and females. *Endocrinology*, *141*(8), 2951–62. http://doi.org/10.1210/endo.141.8.7622

Sharma, P. K., & Thakur, M. K. (2006). Expression of estrogen receptor (ER) alpha and beta in mouse cerebral cortex: effect of age, sex and gonadal steroids. *Neurobiology of Aging*, *27*(6), 880–7. http://doi.org/10.1016/j.neurobiolaging.2005.04.003

Shughrue, P. J., Lane, M. V, & Merchenthaler, I. (1997). Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *The Journal of Comparative Neurology*, *388*(4), 507–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9388012

Shulman, L. M., & Spritzer, M. D. (2014). Changes in the sexual behavior and testosterone levels of male rats in response to daily interactions with estrus females. *Physiology & Behavior*, *133*, 8–13. http://doi.org/10.1016/j.physbeh.2014.05.001

Simerly, R. B., Swanson, L. W., Chang, C., & Muramatsu, M. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: An in situ

hybridization study. *The Journal of Comparative Neurology*, *294*(1), 76–95. http://doi.org/10.1002/cne.902940107

Simmons, D. A., & Yahr, P. (2011). Nitric oxidergic cells related to ejaculation in gerbil forebrain contain androgen receptor and respond to testosterone. *The Journal of Comparative Neurology*, *519*(5), 900–915. http://doi.org/10.1002/cne.22557

Simpson, E. R. (1998). Genetic mutations resulting in estrogen insufficiency in the male. *Molecular and Cellular Endocrinology*, *145*(1–2), 55–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9922099

Smith, E. R., Stefanick, M. L., Clark, J. T., & Davidson, J. M. (1992). Hormones and sexual behavior in relationship to aging in male rats. *Horm. Behav.*, *26*(1), 110–135.

Smith, J. T. (2013). Sex steroid regulation of kisspeptin circuits. *Advances in Experimental Medicine and Biology*, *784*, 275–95. http://doi.org/10.1007/978-1-4614-6199-9_13

Srivastava, D. P., & Penzes, P. (2011). Rapid estradiol modulation of neuronal connectivity and its implications for disease. *Frontiers in Endocrinology*, *2*, 77. http://doi.org/10.3389/fendo.2011.00077

Srivastava, D. P., Woolfrey, K. M., Woolfrey, K., Jones, K. A., Shum, C. Y., Lash, L. L., … Penzes, P. (2008). Rapid enhancement of two-step wiring plasticity by estrogen and NMDA receptor activity. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(38), 14650–5. http://doi.org/10.1073/pnas.0801581105

Stanić, D., Dubois, S., Chua, H. K., Tonge, B., Rinehart, N., Horne, M. K., & Boon, W. C. (2014). Characterization of aromatase expression in the adult male and female mouse brain. I. Coexistence with oestrogen receptors α and β, and androgen receptors. *PloS One*, *9*(3), e90451. http://doi.org/10.1371/journal.pone.0090451

Susswein, A. J., Katzoff, A., Miller, N., & Hurwitz, I. (2004). Nitric oxide and memory. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *10*(2), 153–62. http://doi.org/10.1177/1073858403261226

Swaney, W. T., Dubose, B. N., Curley, J. P., & Champagne, F. A. (2012). Sexual experience affects reproductive behavior and preoptic androgen receptors in male mice. *Hormones and Behavior*, *61*(4), 472–8. http://doi.org/10.1016/j.yhbeh.2012.01.001

Szymczak, S., Kalita, K., Jaworski, J., Mioduszewska, B., Savonenko, A., Markowska, A., … Kaczmarek, L. (2006). Increased estrogen receptor beta expression correlates with decreased spine formation in the rat hippocampus. *Hippocampus*, *16*(5), 453–63. http://doi.org/10.1002/hipo.20172

Tabatadze, N., Sato, S. M., & Woolley, C. S. (2014). Quantitative analysis of long-form aromatase mRNA in the male and female rat brain. *PloS One*, *9*(7), e100628. http://doi.org/10.1371/journal.pone.0100628

Takahashi, K., Bergstrfm, M., Frndberg, P., & Vesstrfm, E. (2006). Imaging of aromatase distribution in rat and rhesus monkey brains with [11 C] vorozole. *Nuclear Medicine and Biology*, *33*, 599–605. http://doi.org/10.1016/j.nucmedbio.2006.03.009

Tan, X., Dai, Y., Wu, W., Kim, H., Barros, R. P. A., Richardson, T. I., … Gustafsson, J.-Å. (2012). Reduction of dendritic spines and elevation of GABAergic signaling in the brains of mice treated with an estrogen receptor β ligand. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(5), 1708–12. http://doi.org/10.1073/pnas.1121162109

Taziaux, M., Keller, M., Bakker, J., & Balthazart, J. (2007). Sexual behavior activity tracks rapid changes in brain estrogen concentrations. *J. Neurosci.*, *27*, 6563–6572.

Thakur, M. K., & Sharma, P. K. (2007). Transcription of estrogen receptor alpha and beta in mouse cerebral cortex: effect of age, sex, 17beta-estradiol and testosterone. *Neurochemistry International*, *50*(2), 314–21. http://doi.org/10.1016/j.neuint.2006.08.019

Thakur, M. K., & Kumar, R. C. (2007). 17Beta-estradiol modulates age-dependent binding of 40 kDa nuclear protein to androgen receptor promoter in mouse cerebral cortex. *Biogerontology*, *8*(5), 575–82. http://doi.org/10.1007/s10522-007-9102-x

Unger, E. K., Burke, K. J., Yang, C. F., Bender, K. J., Fuller, P. M., & Shah, N. M. (2015). Medial amygdalar aromatase neurons regulate aggression in both sexes. *Cell Reports*, *10*(4), 453–62. http://doi.org/10.1016/j.celrep.2014.12.040

van den Beld, A. W., de Jong, F. H., Grobbee, D. E., Pols, H. A., & Lamberts, S. W. (2000). Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. *The Journal of Clinical Endocrinology and Metabolism*, *85*(9), 3276–82. http://doi.org/10.1210/jcem.85.9.6825

Veldhuis, J. D., & Dufau, M. L. (1993). Steroidal regulation of biologically active luteinizing hormone secretion in men and women. *Human Reproduction (Oxford, England), 8 Suppl 2*, 84–96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8276976

Veldhuis, J. D., Urban, R. J., & Dufau, M. L. (1994). Differential responses of biologically active luteinizing hormone secretion in older versus young men to interruption of androgen negative feedback. *The Journal of Clinical Endocrinology and Metabolism*, *79*(6), 1763–70. http://doi.org/10.1210/jcem.79.6.7989483

Veldhuis, J. D., Zwart, A., Mulligan, T., & Iranmanesh, A. (2001). Muting of androgen negative feedback unveils impoverished gonadotropin-releasing hormone/luteinizing hormone secretory reactivity in healthy older men. *The Journal of Clinical Endocrinology and Metabolism*, *86*(2), 529–35. http://doi.org/10.1210/jcem.86.2.7200

Veldhuis, J. D., Iranmanesh, A., & Mulligan, T. (2005). Age and testosterone feedback jointly control the dose-dependent actions of gonadotropin-releasing hormone in healthy men. *The Journal of Clinical Endocrinology and Metabolism*, *90*(1), 302–9. http://doi.org/10.1210/jc.2004-1313

Vermeulen, A., Kaufman, J. M., Goemaere, S., & van Pottelberg, I. (2002). Estradiol in elderly men. *The Aging Male : The Official Journal of the International Society for the Study of the Aging Male*, *5*(2), 98–102. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12198740

Wagner, C. K., & Morrell, J. I. (1996). Distribution and steroid hormone regulation of aromatase mRNA expression in the forebrain of adult male and female rats: a cellular-level analysis using in situ hybridization. *The Journal of Comparative Neurology*, *370*(1), 71–84. http://doi.org/10.1002/(SICI)1096-9861(19960617)370:1<71::AID-CNE7>3.0.CO;2-I

Walf, A. A., Koonce, C. J., & Frye, C. A. (2008). Estradiol or diarylpropionitrile decrease anxiety-like behavior of wildtype, but not estrogen receptor beta knockout, mice. *Behavioral Neuroscience*, *122*(5), 974–81. http://doi.org/10.1037/a0012749

Walf, A. A., Paris, J. J., & Frye, C. A. (2009). Nociceptive and Anxiety-Like Behavior in Reproductively Competent and Reproductively Senescent Middle-Aged Rats. *Gender Medicine*, *6*, 235–246. http://doi.org/10.1016/j.genm.2009.03.002

Walker, D. M., Kermath, B. A., Woller, M. J., & Gore, A. C. (2013). Disruption of Reproductive Aging in Female and Male Rats by Gestational Exposure to

Estrogenic Endocrine Disruptors. *Endocrinology*, *154*(6), 2129–2143. http://doi.org/10.1210/en.2012-2123

Wersinger, S. R., & Rissman, E. F. (2000). Dopamine activates masculine sexual behavior independent of the estrogen receptor alpha. *J. Neurosci.*, *20*, 4248–4254.

Winslow, J. T., & Insel, T. R. (2004). Neuroendocrine basis of social recognition. *Current Opinion in Neurobiology*, *14*(2), 248–53. http://doi.org/10.1016/j.conb.2004.03.009

Winters, S. J., & Troen, P. (1986). Testosterone and estradiol are co-secreted episodically by the human testis. *The Journal of Clinical Investigation*, *78*(4), 870–3. http://doi.org/10.1172/JCI112673

Wood, R. I., & Newman, S. W. (1993). Mating activates androgen receptor-containing neurons in chemosensory pathways of the male Syrian hamster brain. *Brain Research*, *614*(1–2), 65–77. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8348332

Wood, R. I. (1996). Estradiol, but not dihydrotestosterone, in the medial amygdala facilitates male hamster sex behavior. *Physiology and Behavior*, *59*(4–5), 833–841.

Wood, R. I., & Newman, S. W. (2008). Androgen and Estrogen Receptors Coexist within Individual Neurons in the Brain of the Syrian Hamster. *Neuroendocrinology*, *62*(5), 487–497. http://doi.org/10.1159/000127039

Wood, R. I., & Williams, S. J. (2001). Steroidal control of male hamster sexual behavior in Me and MPOA : effects of androgen dose and tamoxifen. *Physiology & Behavior*, *72*, 727–733.

Wu, D., & Gore, A. C. (2010). Changes in androgen receptor, estrogen receptor alpha, and sexual behavior with aging and testosterone in male rats. *Hormones and Behavior*, *58*(2), 306–16. http://doi.org/10.1016/j.yhbeh.2010.03.001

Wu, D., & Gore, A. C. (2009). Sexual experience changes sex hormones but not hypothalamic steroid hormone receptor expression in young and middle-aged male rats. *Hormones and Behavior*, *56*(3), 299–308. http://doi.org/10.1016/j.yhbeh.2009.06.007

Wu, D., Lin, G., & Gore, A. C. (2009). Age-related changes in hypothalamic androgen receptor and estrogen receptor alpha in male rats. *The Journal of Comparative Neurology*, *512*(5), 688–701. http://doi.org/10.1002/cne.21925

Xiao, K., Chiba, A., Sakuma, Y., & Kondo, Y. (2015). Transient reversal of olfactory preference following castration in male rats: Implication for estrogen receptor involvement. *Physiology & Behavior*, *152*, 161–167. http://doi.org/10.1016/j.physbeh.2015.09.016

Xu, S., Yang, Y., Tao, W., Song, Y., Chen, Y., Ren, Y., … Pang, D. (2012). Tamoxifen adherence and its relationship to mortality in 116 men with breast cancer. *Breast Cancer Research and Treatment*, *136*(2), 495–502. http://doi.org/10.1007/s10549-012-2286-z

Xue, H., Gai, X., Sun, W., Li, C., & Liu, Q. (2014). Morphological changes of gonadotropin-releasing hormone neurons in the rat preoptic area across puberty. *Neural Regeneration Research*, *9*(13), 1303–12. http://doi.org/10.4103/1673-5374.137578

Yamaguchi, N., & Yuri, K. (2012). Changes in oestrogen receptor-β mRNA expression in male rat brain with age. *Journal of Neuroendocrinology*, *24*(2), 310–8. http://doi.org/10.1111/j.1365-2826.2011.02231.x

Yeap, B. B., Alfonso, H., Chubb, S. A. P., Handelsman, D. J., Hankey, G. J., Norman, P. E., & Flicker, L. (2012). Reference ranges and determinants of testosterone, dihydrotestosterone, and estradiol levels measured using liquid chromatography-tandem mass spectrometry in a population-based cohort of older men. *The Journal of Clinical Endocrinology and Metabolism*, *97*(11), 4030–9. http://doi.org/10.1210/jc.2012-2265

Yeap, B. B., Knuiman, M. W., Divitini, M. L., Handelsman, D. J., Beilby, J. P., Beilin, J., … Hung, J. (2014). Differential associations of testosterone, dihydrotestosterone and oestradiol with physical, metabolic and health-related factors in community-dwelling men aged 17-97 years from the Busselton Health Survey. *Clinical Endocrinology*, *81*(1), 100–8. http://doi.org/10.1111/cen.12407

Yeh, K.-Y., Pu, H.-F., Wu, C.-H., Tai, M.-Y., & Tsai, Y.-F. (2009). Different subregions of the medial preoptic area are separately involved in the regulation of copulation and sexual incentive motivation in male rats: a behavioral and morphological study. *Behavioural Brain Research*, *205*(1), 219–25. http://doi.org/10.1016/j.bbr.2009.06.025

Yin, W., Maguire, S. M., Pham, B., Garcia, A. N., Dang, N.-V., Liang, J., … Gore, A. C. (2015). Testing the Critical Window Hypothesis of Timing and Duration of Estradiol Treatment on Hypothalamic Gene Networks in Reproductively Mature and Aging Female Rats. *Endocrinology*, *156*(8), 2918–33. http://doi.org/10.1210/en.2015-1032

Yun, H. Y., Gonzalez-Zulueta, M., Dawson, V. L., & Dawson, T. M. (1998). Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(10), 5773–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9576960

Zhao, C., Fujinaga, R., Yanai, A., Kokubu, K., Takeshita, Y., Watanabe, Y., & Shinoda, K. (2008). Sex-steroidal regulation of aromatase mRNA expression in adult male rat brain: a quantitative non-radioactive in situ hybridization study. *Cell and Tissue Research*, *332*(3), 381–91. http://doi.org/10.1007/s00441-008-0606-8

Zhao, L., & Brinton, R. D. (2007). Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. *Brain Research*, *1172*, 48–59. http://doi.org/10.1016/j.brainres.2007.06.092

Zhu, Z., Liu, X., Senthil Kumar, S. P. D., Zhang, J., & Shi, H. (2013). Central expression and anorectic effect of brain-derived neurotrophic factor are regulated by circulating estradiol levels. *Hormones and Behavior*, *63*(3), 533–42. http://doi.org/10.1016/j.yhbeh.2013.01.009